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THE PARATHYROID GLANDS

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Since, in the past decade, a number of reviews have discussed calcium and phosphorus metabolism in general (485, 15, 256, 90, 400) and the physiology of the parathyroid glands in particular (467, 110, 137, 337, 404, 13) from various points of view, we propose in these pages to devote our attention chiefly to those aspects of the subject in which our knowledge has recently advanced, and on which attention is at present focussed. There appears to be no necessity for yet another survey of the first contributions to the subject and the older literature, which we have therefore largely ignored, especially in cases where improvement in experimental technique or analytical methods have cast doubt on the validity of the results reported. We intend also to restrict our survey to the relation of the parathyroid glands to the metabolism of calcium and phosphorus. Thompson (501, 502) has recently obtained parathyroid extracts which have no influence on the level of the serum calcium, and which powerfully retard growth; but since similar extracts may be obtained from other tissues (62), this topic hardly requires discussion here.

The effects of extirpation of the parathyroid glands. Since the experiments of Gley in 1891 it has been generally recognized that extirpation of the parathyroid glands of the dog leads, usually within two or three days, to the characteristic manifestations of tetany. There is a prompt and rapid fall in the concentration of calcium in the serum (329); this, however, is not due to increased calcium excretion, as was thought at one time (329, 440), since in fact the urine calcium is reduced (114, 181, 281), and the fecal calcium reduced (481) or only very slightly increased (181). More striking than the change in calcium excretion is the reduced excretion of phosphorus (181, 184); yet the increase in the inorganic phosphorus content of the blood is relatively slight (184,

531, 150), and may indeed escape detection (189, 440). The content of serum in sodium, potassium and magnesium is unchanged (531), and the excretion of magnesium may be slightly increased (114, 181), while that of sodium and potassium is possibly slightly decreased (187); eventually anuria may develop. The calcium content of muscle is slightly decreased (324, 247) or unchanged (34, 133). Eventually fine muscular tremors, probably ascribable to the low concentration of calcium ions in the medium, set in, and lead to a rise in body temperature (410, 75) which in turn frequently produces hyperpnea (110, 82, 75)—the excitability of the respiratory centre being perhaps abnormally high—and the resulting alkalosis leads to violent convulsions. These convulsions are arrested by any increase in hydrogen-ion concentration, even that produced by the muscular contractions themselves or by breathing CO_2 (493), but inevitably recur unless an acidosis can be maintained experimentally as in uranium nephritis (494).

Tetany can be brought on by raising the dog's temperature artificially and thus producing hyperpnea (75), though the resulting alkalosis would not be sufficient to produce tetany in dogs having a normal serum calcium; similarly, cooling the animal reduces the severity of the spasms though the fibrillary twitchings of the muscles remain (75). There is no definite level of the serum calcium at which tetany may be expected (159, 327, 190, 468); it may be present with a serum calcium of 8.7 mgm. per cent (82), or absent at 3.5 (369). This is only to be expected, since calcium is present in serum in more than one form. The rapidity with which the decrease in calcium takes place may be a factor (186, 150), and the ratio of calcium to inorganic phosphate in the serum is no doubt of some significance (410, 165). Death finally results from exhaustion or from asphyxia due to respiratory spasm. There is insufficient evidence to decide what part of the neuromuscular apparatus is chiefly affected in tetany; degenerative changes in motor neurones have been described (139) but, as Loeb showed long ago, even isolated muscles are sensitive to lack of calcium.[1] There is very marked variation between individuals in the rapidity of onset and the severity of the symptoms. Oddly enough, little attention has been paid to the occurrence of accessory parathyroid tissue in the dog; in one series of thirty-three animals it was found only once (410), but it has been concluded on physiological grounds that it is probably more often present than absent

[1] The demonstration by H. Hartridge and R. West (1931, Brain, liv, 312) that small doses of curare abolish parathyroid tetany evidently suggests that the convulsions are of nervous rather than muscular origin.

(186). Taylor (498) has summarized the scanty data in the literature bearing on this question, and inclines to a similar conclusion.

More adequate histological studies have been made in cats, and it appears from them that accessory parathyroid tissue is exceedingly common (152, 452, 375), especially within the thymus. On the other hand only a few physiological studies of parathyroidectomized cats have been made. Nicholas and Swingle (375) found the train of symptoms to be essentially the same as in the dog, if somewhat less rapid in sequence. Absence of severe tetany could be correlated with the presence of accessory parathyroids in several instances. They noted that in tetany the nictitating membrane is drawn laterally over the surface of the eye, simulating external strabismus. Taylor and Fine (496), however, only rarely saw frank tetany or great decreases in serum calcium; they believed that calcium excretion from the intestine was increased. In the sheep (467) and goat (303) tetany is comparatively rare. The rabbit, in spite of the frequent occurrence of accessory parathyroids (337), succumbs rather rapidly to a particularly violent and intractable tetany, in which the increase in serum inorganic phosphate is often more striking than the decrease in serum calcium (108, 165).

Accessory parathyroids were demonstrated only in ten per cent of a series of rats (242) yet parathyroidectomy is rarely fatal in this species. Tweedy and Chandler (513) noted a fall in serum calcium to 5–8 mgm. per cent, with a return to normal values in three to nine months. They did not see definite symptoms of tetany, as earlier workers did (148, 152, 271). Accordingly, this species has been used for the study of the effects of prolonged deprivation of parathyroid hormone. Erdheim (148) noted that the teeth became opaque, brittle, and distorted, and this has been confirmed many times (507, 234, 286, 152); there is a general disorganization of the enamel, and exostoses of the alveolar bone occur (271). The bones are to some extent decalcified (148) in appearance, and on analysis are found to be low in ash, calcium, and phosphorus, though relatively high in magnesium (202). Cataractous changes in the lens have been noted (147), but a general increase in the calcium content of the muscles and viscera (311) is not established (12).[2]

[2] We consider that the calcium metabolism of the parathyroidectomized animal requires further study. In an important paper which reached us too late to be incorporated into this review, E. Bulbring (1931, Arch. exp. Path. u. Pharm., clxii, 209) reports that the bodies of parathyroidectomized rats contain more calcium and phosphorus than the controls, this excess being traced in part to the skeletal muscle, expecially on calcium-rich diet, and not to the bone.

Waggener (523) has recently noted that in the frog removal of the parathyroids leads in less than two days to violent tetanic symptoms, spastic paralysis, a fall in blood calcium, and presently death with stiffened, outstretched limbs. We feel that this discovery makes it highly improbable that any mammal can escape tetany after ablation of the *whole* of the parathyroid tissue, unless special treatment is promptly instituted; and it is to be considered that treatment efficacious after ordinary thyroparathyroidectomy might not improbably fail in this ideal case, as Marine suggested long ago.

The rôle of changes in concentration of hydrogen-ion and of inorganic phosphate in the plasma, in the appearance and disappearance of tetany, requires some consideration. It is commonly supposed, first, that only ten or twenty per cent of the total serum calcium is actually present as calcium ion, and secondly, that serum behaves as if it were in equilibrium with solid calcium phosphate. The experimental evidence supporting these hypotheses will be examined later; for the present, they may be accepted, since nothing in our knowledge of experimental or clinical tetany speaks against them. It follows from the second hypothesis that any increase in the concentration of PO_4''' ions in the plasma will decrease the amount of $Ca^{\cdot\cdot}$ ion; a decrease in hydrogen-ion concentration will increase the PO_4''' ions at the expense of H_2PO_4' and HPO_4'', which are not relevant in this question, since plasma is certainly not saturated with respect to $CaHPO_4$ (456); at constant pH, an increase in total inorganic phosphate will doubtless increase PO_4''' ions with the rest. Hence we may, if we wish, logically suppose that tetany depends solely on the falling of the $Ca^{\cdot\cdot}$ ion concentration below some definite threshold, and explain the action of changes in hydrogen-ion or phosphate-ion concentration through the mechanism just discussed. If we further suppose that the proportion of the total serum calcium which exists as ions is not a strictly fixed one, the theory becomes practically safe from experimental attack! There is, however, no guarantee that changes in hydrogen-ion concentration may not *directly* influence neuro-muscular excitability, perhaps through their effect on the dissociation of oxyhemoglobin (180), nor that increase in PO_4''' ion or even CO_3'' ion is not more directly important than decrease in $Ca^{\cdot\cdot}$ ion concentration. Esau and Stoland (150) suggest, on scanty indirect evidence, that the muscular hyperexcitability is due to changes in phosphocreatine, which secondarily affect the calcium-potassium ratio. Direct evidence (133) does not indicate any change in the phosphorus partition in muscle after parathyroidectomy.

It has long been recognized that the fatal consequences of parathyroidectomy in the dog or cat may be averted by vigorous calcium therapy, as for example, by oral administration of calcium lactate or gluconate (326, 313), or indeed of any suitable calcium-rich diet. The beneficial effect of lactose may depend partly on facilitating the absorption of calcium from the intestine through acid fermentation (40), and partly through the action which, apparently more than other sugars, it has in lowering the blood inorganic phosphate (428). Helpful too is the injection of solutions of calcium salts, even of calcium lactate, the beneficial effect of the calcium ion in this case being greater than the harmful effect of the alkalosis produced as the lactate ion is disposed of (75). Salvesen (440) believed that calcium chloride, injected into cats in tetany, was rapidly and quantitatively excreted by the intestine; but later studies have confirmed him only in part (481) or not at all (496). The beneficial effect of magnesium chloride (327) or lactate (534, 150), or of strontium lactate (492), has been ascribed to the depressant action of these cations on irritable tissues (468, 112, 150), since morphine is similarly helpful. But the low serum calcium makes parathyroidectomized dogs unusually intolerant of magnesium salts (343); it may be that the above-mentioned increase in magnesium excretion is a compensatory mechanism. The withdrawal of rather large amounts of blood and injection of saline is beneficial (325); this effect, at one time regarded as supporting the theory that tetany is produced by a circulating toxic substance, is now thought to depend on a raising of the concentration of calcium in the blood (491), which may also be seen in normal dogs (393), and is doubtless comparable to the calcium-raising effect of asphyxia (111). But while this effect of asphyxia has been noted in several species, the effect of hemorrhage in the rabbit is to lower the serum calcium (94, 481), or to leave it unchanged (359), while in the cat there is again a decrease (223) or no effect (481). The administration of ammonium chloride has also been found to relieve tetany (61, 535, 187); the acidosis produced is no doubt helpful directly, and indirectly valuable in increasing phosphate excretion. More certain than any of these methods of treatment, however, is the use of active extracts of the parathyroid gland, daily injections of which permit survival, apparently indefinitely, of completely thyroparathyroidectomized dogs and cats (103, 105).

Various types of tetany. Recent reviews of the various forms of tetany met with clinically are available (193, 218). The accidental removal by operation of a greater or lesser amount of parathyroid tissue has effects,

in human patients, which seem to be quite analogous to experimental parathyroid tetany in dogs. The discovery of methods for the preparation of active extracts of the glands has provided the clinician with a powerful specific for use in emergencies and in severe cases. Many striking results have been reported (102, 117, 365, 254, 229, 315, 316, 145) and the method is now in daily use in many countries. It appears that the parathyroid hormone, like insulin, is best used as a supplement and a reserve in conjunction with other forms of treatment—in this case a diet rich in calcium, and with sufficient vitamin D to ensure sufficient absorption of calcium and phosphorus. Infantile tetany is also associated with a low serum calcium, sometimes with a high plasma inorganic phosphate (463), and again the action of parathyroid extract, though less frequently called for, is beneficial (317). In the rather rare cases where no obvious cause can be found for the occurrence of low serum calcium and tetany, and dietary factors can be excluded, a diagnosis of idiopathic hypoparathyroidism has been made; and in such cases, not unnaturally, parathyroid extract is used with success (127, 317, 5). The theory that epilepsy is accompanied by low serum calcium and alkalosis, both factors tending to increase neuromuscular excitability, does not seem to be in accordance with the evidence (310, 387); in chorea, too, the observed lowering of the serum calcium seems too slight to be significant (527).

Cataract has frequently been noted in the eyes of parathyroidectomized dogs (140, 326, 136) and rats (147), and has been noted in idiopathic hypoparathyroidism in man (5). It is a common accompaniment of post-operative tetany; Cole (98) collected 38 such cases. He believes that more use of parathyroid extract and less use of high-calcium diets would reduce the incidence and severity of these cataracts; but it is unfortunately not clear that general experience sanctions this optimistic attitude. Cataractous lenses are distinguished by their high calcium content (78, 2); but the view that senile cataract is regularly accompanied by latent tetany and low serum calcium (158) cannot be supported (510, 406, 2). It has also been suggested that otosclerosis is usually accompanied by latent tetany (162, 23) and low serum calcium (241, 308) and that parathyroidectomized dogs may rapidly become deaf (137). Aub (15) and Mirvish (364) were unable to satisfy themselves that sufferers from otosclerosis had low serum calcium, but believed that in some cases hearing was slightly improved by treatment with parathyroid hormone.

One serious disadvantage of the present parathyroid extracts as

therapeutic agents is that many subjects become immune to their action. This has also been noted in dogs. In such cases the serum calcium may still be raised by other means, as by acidosis (4). It appears to be very improbable that the body should develop immunity to one of its own internal secretions, and we have preferred the view that the phenomenon is due to the potent extracts used having been somehow altered in the rather drastic treatment involved in their preparation, and that persons immune to the extracts may respond to the secretion from their own glands. Thus, for example, Aub (4) reports the case of a boy whose serum calcium, in a prolonged course of treatment with parathyroid extract, fell from a maximum of 12.5 mgm. per cent to 9.5 mgm. per cent. At this time the treatment was interrupted for four months, but tetany was not reported, and at the end of this interval the serum calcium was 10.2. When the injections were resumed the calcium steadily decreased, suggesting that the immunity persisted (in other cases it has lasted over a year) and possibly that the injections checked the secretion of the hormone from the patient's own parathyroids. The low-calcium diet on which he was placed when in hospital, may, however, have had something to do with this.

Tetany may be induced experimentally by voluntary over-ventilation of the lungs (100, 173). The essential change in blood chemistry is the uncompensated CO_2 deficit and consequent increase in pH. The serum calcium is normal (192, 239) or even slightly elevated (173, 480); the serum inorganic phosphate is normal (192) or low (239, 196). Quite similar, undoubtedly, is the tetany which may follow the ingestion of large doses of sodium bicarbonate, or the "gastric" tetany seen when the body loses large amounts of acid in persistent vomiting; in neither case is there reason to suppose that the serum calcium is lowered (504).

In the tetany which may result when sodium phosphate is injected into animals (47, 109, 59, 165) or administered orally in large doses (443), several factors may play a part. Since sodium chloride may have a similar effect (101, 180) some workers have spoken of a specific tetany-producing effect of the sodium ion in excess, or of high sodium-calcium ratios; but the effect of the phosphate is greater than that of other sodium salts (47). Secondly, there is no doubt a decrease in calcium-ion concentration, and indeed a decrease in total serum calcium is observed (47, 200, 386). Thirdly, there is the effect of the reaction, since tetany is observed only when the phosphate solutions injected are of pH 7.3 or greater; acid sodium phosphate has no tendency to produce tetany, though it lowers serum calcium (47, 109) and greatly

increases calcium excretion (182, 59, 263, 77). Farquharson, Salter and Aub (154) do not find that the phosphate ions in themselves have any effect on calcium excretion in human subjects.

A similar but possibly simpler case is that of the tetany seen in rats during the healing of rickets, which is of particular interest from its probable close relationship to infantile and adolescent tetany. Rats made rachitic on a diet of the usual type, high in calcium and low in phosphorus, very commonly show tetany along with healing of the bone when the phosphorus content of the diet is suitably increased (275) or the calcium content lowered (221), or even when vitamin D is added (464); in the two latter cases the absorption of phosphorus from the intestines doubtless becomes more efficient. Similar observations have been made on rabbits (200). Merely fasting the rats may lead to tetany and healing of the rachitic lesions (92, 539), possibly because phosphorus is set free and made available as protoplasm is consumed. In all such cases there is a sharp change, at times even an inversion, in the abnormally high calcium-to-phosphate ratio of the serum, and to this the tetany must be ascribed, since the hydrogen-ion concentration of the plasma is moderately increased (466).

Many workers, at different times, have been impressed by the similarity between the tetanic convulsions of guanidine poisoning and those which follow parathyroidectomy, and have postulated a close relation between the two syndromes. In its latest form (160) the theory suggests that in the absence of the parathyroids dimethylguanidine is produced in the body from creatinine, and in some way reduces or inactivates the ionized calcium of the serum. Another recent version (371) ascribes a rôle to the "volatile chlorine" of the blood, which however appears to be a myth. Though it now appears that methylguanidine may be isolated from muscle by methods which exclude its formation during extraction (285, 469), the evidence formerly forthcoming to show that the content of blood and urine in guanidine and its derivatives was increased after parathyroidectomy is now known to be based on untrustworthy analytical methods (333). While some observers have reported a lowered serum calcium in guanidine poisoning (528, 191), the majority have found that the reverse is true (38, 372, 105, 358); and the methods used in demonstrating a reduction in the ionized calcium (31) are not dependable. The similarities between the two syndromes, though striking, are not complete (143, 489, 38); fatty degeneration of the liver is typical of guanidine poisoning, where the general congestion and hyperemia of the viscera which follows para-

thyroidectomy is not seen; in guanidine poisoning there is a continued steady rise of blood urea and non-protein-nitrogen, after the first hour or two, and no such rise is seen after parathyroidectomy until the animal is moribund (107, 333). In spite of the fact that calcium has some therapeutic value in guanidine poisoning (125), parathyroid extracts, potent in replacement therapy, do not protect the dog (105), cat (215), or mouse (488) against guanidine. Recent work (125, 358) ascribes to the liver, rather than to the parathyroids, the chief rôle in preventing dangerous accumulations of guanidine-like substances in the body; the evidence of histological changes in the parathyroids after guanidine poisoning (487) proves very little. The more recent work on experimental and clinical hyperparathyroidism offers overwhelming proof of a *direct* relationship between the parathyroids and calcium (or phosphorus) metabolism. Against the suggestion that toxic substances from the intestine are important in parathyroid tetany may be set the demonstration that colectomy does not modify the symptoms (384, 511).

The effect of parathyroid extracts in normal animals. The most striking and conspicuous result of the subcutaneous or intramuscular injection of a potent parathyroid extract in a normal dog is the rise in the serum calcium. The amount of this rise is in general proportional to the dose, except that rises above 18 mgm. per cent are seldom observed (105); the rise is gradual, and attains its maximum in twelve to eighteen hours. These points will be discussed again in connection with the technique of assay. The effect may also be obtained in anesthesia (105), although the administration of ether is said to lower the blood calcium (96) despite the fact that asphyxia increases it. There is no significant effect upon the blood pressure (105), though there may be some slowing of the pulse (251). Vomiting may occur, but generally speaking the animal shows no ill effects and recovers completely; the time elapsing before the serum calcium returns to normal depends upon the amount by which it was increased, the slope of the descending curve being similar to that of the ascending curve (104). A diuretic action has been noted (251, 495). There may be some lowering of the alkali reserve (382), but the suggestion that the mobilization of calcium is due merely to an acidosis cannot be accepted. A slight rise in serum potassium (382, 35) and magnesium (444) has been reported; the latter effect lasts only two or three hours, and has vanished before the rise in calcium approaches its maximum (105). Doubtless there is sometimes a decrease in plasma inorganic phosphate, though acidosis may mask the calcium-phosphate

relationship discussed above, and it must be said that at times, certainly, not only total inorganic phosphate but even PO_4''' ions may increase. Intravenous injection causes the serum calcium to rise more rapidly, so that the peak is attained in from four to eight hours (382, 9), but the rise is only half that seen with equal doses intramuscularly (9). Injection into the spinal fluid is also effective in most cases (378), which contrasts with the behaviour of insulin. The impression gained in the first studies (103) that oral administration was effective has proved to be misleading, the results being highly irregular.

The response of the human subject appears to be similar to that of the dog, and the time curve of the serum calcium is similar (215). Intravenous injections may lead to dramatic improvement in severe postoperative tetany within half an hour (145). There may be an increased hydrogen-ion concentration in the plasma (64) with no measurable alteration in the carbon-dioxide combining power (64, 85, 5). The fall in plasma inorganic phosphate is at least as rapid, though not so great, as the rise in calcium (64, 5). An increase in the excretion of calcium and phosphorus in the urine, and in the volume of urine secreted, may be seen in the first hour (5), before there is any great change in the composition of the blood.

The literature is full of contradictions in its reference to the response of cats injected with parathyroid extracts. Stewart and Percival (481) were able to demonstrate a rise of 3 mgm. in serum calcium in the fifteenth hour after the subcutaneous injection of ten units; but after intravenous injection into anesthetized or decerebrate cats they found higher values at the second hour than at the third. Thölldte (500) has made similar claims, and Herxheimer (215) maintains that the serum calcium may attain its maximum in ten minutes! A careful re-examination of this point in our laboratory (9) failed to reveal any regular increase in the cat's serum calcium in the first two hours after injection. Others again have had difficulty in obtaining regular results with cats (17), and Bauer, Aub, and Albright go so far as to say that they "have never been able to demonstrate a rise in serum calcium in cats or kittens after parathormone administration" (25). We can find no clue to any explanation of these divergences of opinion; whether differences in diet, or in race, may be invoked to reconcile these data is a question for the future. It is likely that in cases where a rise in serum calcium is obtained, the accompanying changes observed in other species will also occur in the cat, but little work has been done on these lines. It has been noted that the excretion of calcium by the intestine is little affected

by injections of parathyroid hormone (481, 496). It is of importance, in connection with the theory of the action of the hormone, that hypercalcemia may be produced in *eviscerated* cats (481, 498).

In our experience the rabbit is a most unsatisfactory animal for studies involving determination of the serum calcium, since the values obtained show very great individual variations; we believe that the normal is perhaps less definitely fixed in this species than in any other of the ordinary laboratory animals. It will be noted in several places in this review that certain experimental procedures may alter the serum calcium level in the rabbit and apparently have no such effect in other species. Yet the parathyroid hormone, so effective elsewhere, has very limited potency. That a rise in serum calcium may usually be obtained with relatively enormous doses (50–100 units) appears to be certain (108, 128, 370, 195, 257); but we are less inclined to accept as a general rule the suggestion that the general rise is much more rapid than in the dog (215, 500, 56) or that 20 units may regularly give a 2–3 mgm. per cent increase (56, 257). The German workers who insist upon the specific nature of the serum-calcium time-curve after parathyroid administration in the cat, rabbit and dog, believe also that there are parallel differences in the rate of disposal of calcium injected into the blood stream (56, 500).

Very striking are the consequences of repeated injections of parathyroid hormone in the dog, in dosages such as 25 units every four hours. The serum calcium rises rapidly and attains a value of about 20 mgm. per cent in perhaps 20 hours, to remain at this value for some time. In spite of continued administration of the extract, however, it begins at about the 36th hour to decline steadily (104, 105). It will be realized that the times quoted are those of typical experiments, and that there may be much individual variation in susceptibility. The excretion of calcium, phosphorus, and nitrogen in the urine increases promptly when the experiment begins, and declines almost as sharply if the experiment is interrupted; the excretion of calcium in the feces is also increased, though relatively less, and that of phosphorus is only slightly affected; the ratio of nitrogen to phosphorus in the urine decreases, showing that the extra phosphorus is not wholly derived from protein (183). At about the twenty-fourth hour the non-protein nitrogen and the urea of the blood begin to rise, and increase steadily throughout the experiment from that time onward; the plasma inorganic phosphate, which at first may decrease slightly, later increases in a curve which runs closely parallel to that of the blood urea; it is therefore likely that those increases

are both to be attributed to failing renal function, especially since it appears that the urinary excretion of phosphorus decreases at this time. In fact, little or no urine is formed in the later stages of the experiment. Urgent symptoms of distress begin to appear at about the time when the serum calcium curve turns downwards (104, 495). The fall in calcium is probably due to restriction of calcium solubility by the rising phosphate. Anorexia, diarrhea, vomiting, lassitude, are followed by muscular atony, coma, and death. The blood becomes very viscous; it is increasingly difficult to obtain samples of blood, and difficult to separate serum. The blood volume, as estimated from the hemoglobin concentration by the acid hematin method, decreases very markedly, sometimes after an initial increase, and there can be no doubt that the cell count is much increased; the chloride content of whole blood decreases, as would be expected from the relative diminution of plasma volume. The osmotic pressure of the blood, measured cryoscopically, increases; there is every sign of dehydration and of failing circulation. Terminally, a marked acidosis sets in (104, 105, 249). Even in the early stages, an increase in the titratable acidity and ammonia content of the urine may be observed to accompany diuresis (495, 64), yet the administration of alkali aggravates the symptoms (367). A decrease in electrical excitability accompanies the rising serum calcium (500). Postmortem examination discloses congestion of the alimentary canal and the presence of blood in the stomach and intestine; the capillaries of the stomach are somewhat dilated, particularly in the superficial area, and much extravasation of blood has taken place, especially from the fundus (343), apparently by diapedesis, since the walls of the vessels are not ruptured (104, 110). In young animals, hemorrhages in the bone marrow are conspicuous (498). The calcium content of the soft parts, and especially of the kidney, appears to have increased (110, 247). Histologically, calcification can be seen in the space of Bowman's capsule and the lumina of the tubules, as well as in the walls of the lesser arteries, the Kuppfer cells of the liver, the walls of the bronchi, and the interstitial connective tissue of the heart (249, 305); these findings strongly support the view that the rise in blood urea and inorganic phosphate are due to failing renal function.

It is possible to imitate this pathological picture by suitable intravenous injections of inorganic salts. The injection of calcium salts leads to a rapid but not quantitative excretion, chiefly by way of the intestine; the calcium content of the viscera is not increased, except in the kidney (434, 282); the difficulty of accounting for all the calcium

injected has led some workers to suppose, not unreasonably, that some may be deposited in the bones (211, 222). In any case, the injected calcium rapidly disappears (440, 109), especially if there is acidosis (282). Large doses of calcium lactate given by oral route may also provoke hypercalcemia, but only exceptionally is the result fatal (228, 109, 304). As already mentioned, the injection of acid sodium phosphate leads to a hyperphosphatemia and hypocalcemia which is well tolerated, while alkaline sodium phosphate (even at the reaction of the plasma) produces tetany. The combined injection of calcium chloride and alkaline sodium phosphate is well tolerated, and the composition of the blood very rapidly returns to normal (182, 109); but with acid sodium phosphate (NaH_2PO_4) and calcium chloride the pathological and biochemical picture of severe parathyroid overdosage may be duplicated almost in its entirety (109). For this and other reasons, to be discussed later especially in connection with the hypercalcemia induced by overdosage with irradiated ergosterol, we are inclined to ascribe the chief rôle in the production of these striking symptoms to the simultaneous presence of hypercalcemia and hyperphosphatemia.

It is much more difficult to produce these symptoms in the cat (500). In the rabbit, enormous doses of the active extracts may produce marked hypercalcemia, but the inorganic phosphate is not significantly increased (108), and the condition of the animal is not seriously affected (330, 495, 370, 108, 500). The rat is resistant also, but is not wholly immune; 200 units, in divided doses, may produce marked hypercalcemia and at times death (437, 513). It has been objected (498) that the amounts of material injected are so large that it is not possible to assert that the rise in serum calcium and the toxic effects are produced by the specific parathyroid hormone. The mouse (488), the guinea pig (330), and the fowl (498, 278) all appear to be highly resistant. It may be that in these species calcium is more readily disposed of, and, possibly because of this, the kidney function is not impaired and the retention of phosphate and of nitrogen compounds does not occur. Calcification does not occur in the kidneys of mice treated with parathyroid extracts (305).

We may turn now to consider the effects of prolonged treatment with parathyroid extracts at sub-lethal levels. Greenwald (185) administered such doses as 10 units a day to dogs, and noted that the excretion of calcium was at first enormously increased, from 0.3 mgm. daily (in the urine) to 39 mgm., after which there was a gradual decline, followed by a sharp drop to subnormal levels when the treatment was suspended. The excretion of calcium and phosphorus in the feces was little affected,

but fell in the recovery period. The urine phosphorus increased over 20 per cent and remained high throughout the experiment, but again fell below normal in the after-period. Nitrogen excretion was little affected. The only conceivable source for the extra calcium excreted was the skeleton, but rarefaction of the bones was not demonstrable by x-ray; the ratio of extra calcium to extra phosphorus was much lower than the calcium-phosphorus ratio of bone. Bischoff (48), however, reports improved calcium retention in rachitic puppies treated with parathyroid. Jaffe and Bodansky (260) were able, by gradually increasing the dose, to give up to 20 units daily to young dogs over six months without serious hypercalcemia; they found that loss of appetite was a reliable indication that the dosage was too great, and that the tolerance was greater when the calcium intake was low. There was marked stunting of growth, and at the end of the experiment they could demonstrate great resorption both of spongy and cortical bone, with fibrosis, new formation of osteoid tissue, and deformation of the limbs—a picture closely approximating to generalized osteitis fibrosa as met with clinically. These results have been confirmed (264). Osteitis fibrosa is not unknown in dogs, and in one case the parathyroids were examined and found not abnormal (532). Thinning of the trabeculae in the bones of cats could not be obtained with parathyroid extracts (25).

Bodansky and Jaffe (51) have carried out similar experiments on guinea pigs, where the danger of overdosage is less. In young, fasted animals a single large dose (100 to 200 units per kilo) may raise serum calcium from the normal level (10.5 mgm. per cent) to as much as 15 mgm. per cent over 24 to 36 hours, and resorption of both cortical and spongy bone can be demonstrated with increasing clearness from the twelfth hour onwards; in older or in recently fed animals such results were not obtained, and a dose of 1000 units per kilo is not fatal. With smaller doses given daily over long periods, a pathological picture quite comparable to generalized osteitis fibrosa can be obtained while the composition of the blood is almost normal. In the rabbit the serum calcium may be maintained at a high level by prolonged treatment with parathyroid extract (353), and the trabeculae of the bones are reduced in number and density (25).

The rat has been much used in experiments of this type, but unfortunately the results are somewhat discordant. In a recent study in this laboratory by Mr. L. I. Pugsley (407) rats on a low calcium diet received twenty units of parathyroid extract daily for fourteen days. There was a prompt and most striking increase in the excretion of calcium in the

urine, which jumped in four days from 0.2 mgm. daily to 6–7 mgm., but in spite of continued treatment fell almost as rapidly as it had risen, back to the original level; the calcium content of the feces was somewhat elevated throughout the treatment, but the phosphorus excretion, which was considerably greater than the calcium excretion, was not demonstrably increased in either channel—a point, it seems to us, of the greatest interest. Since the total "extra calcium" excreted was less than 30 mgm. in these experiments, it is hardly surprising that Day (130), using much smaller doses (15 *Hanson* units daily) was unable to show that the calcium content of the entire body was reduced by parathyroid extracts in a prolonged experiment. Burns (80) was unable to affect calcification in growing rats, but the amount of parathyroid hormone administered was comparatively small; Waltner (524) found in rachitic rats that calcium excretion was increased and the bones, examined by means of x-rays, showed "osteoporosis," and Lambie, Kermack and Harvey (299) were able in a short time to thin the trabeculae of the bones and reduce their ash content, although, curiously, the calcium content was not altered. On the other hand, Bauer, Aub, and Albright (25), in a prolonged experiment, giving 8 units a day on a high-calcium diet, saw no hypercalcemia but found by x-ray that the bones were shorter and *denser* than those of the controls; they confess themselves "unable to explain these findings."[3]

Certainly they are not easy to bring into line with the finding that the administration of parathyroid extract to normal or rachitic children leads to loss of calcium from the body (230, 64), though it has been said that calcium retention in tetany may be improved by treatment with

[3] E. Bulbring (1931, Arch. exp. Path. u. Pharm., clxii, 209) finds that treatment with parathyroid extract reduces the calcium and phosphorus content of the bones, but the effect on the total balance of these elements varies with their representation in the diet. Mr. Pugsley, continuing the experiments described above, was unable to detect the presence of parathyroid hormone in the urine of rats which while still receiving injections, had ceased to show increased calcium excretion; on the other hand, the serum of such animals did not appear to inhibit the usual action of the parathyroid extracts in susceptible rats. But our conception of the nature of this "immunity" has radically changed since Dr. Hans Selye demonstrated to us histological preparations which clearly show that while, in parathyroid-treated rats, there is at first rapid formation of osteoclasts, this is succeeded by formation of osteoblasts and hypercalcification simulating "marble-bone" disease. No suggestion of such reversal of action appears in clinical hyperparathyroidism of long standing, however, and it is possible that it may be a peculiarity either of the extract employed or of the species studied. On the other hand, marble-bone disease should now be reconsidered from this point of view.

parathyroid (317, 231). The action of parathyroid extract on the calcium balance may depend in part upon the calcium and phosphorus content and the acid-base balance of the diet, since the finding that the titratable acidity of the urine and feces of rachitic children is reduced by parathyroid (463) is not otherwise easily reconciled with reports of an increase (64) or no significant change (4, 7) in acid elimination in normal individuals. Hunter and Aub (253) found in adults that the excretion of calcium in the urine was always increased by parathyroid administration, even when the serum calcium was not higher than normal; fecal calcium is only very slightly increased (463, 4, 64) except possibly in rickets (231). In parenthesis, it may be said that this failure of parathyroid to increase fecal calcium has also been noted in calves (427). Albright, Bauer, Ropes and Aub (4) administered parathyroid hormone to normal human subjects on a low calcium intake, and found that the negative calcium balance became still more negative; there was an increase in the excretion of phosphorus in the urine (but not in the feces), and a fall in serum phosphate; however, when the serum calcium rose above 14 or 15 mgm. per cent, the excretion of calcium failed to keep pace, the excretion of phosphorus diminished, and the concentration of inorganic phosphate in the plasma increased. This is in agreement with the view we have already outlined, that the high concentration of calcium in serum and urine may lead, with more or less ease according to species, to renal damage, with retention of nitrogenous waste products and phosphate, and that then, and not till then, the condition of the animal is seriously affected. It has been noted (253) that the serum calcium may rise to 19.8 mgm. per cent for a short time, with no symptoms more serious than slight nausea. Recently, in a courageous experiment, Johnson (264) administered to himself 50 units of parathyroid extract daily, while in calcium equilibrium; he noted increase of calcium and decrease of phosphate in serum, and increase of both in urine; from the 12th day he suffered from severe pains in the bones and joints.

That the parathyroid glands are frequently enlarged in persons suffering from diseases of bone was first recognized by Askanazy in 1904, and has repeatedly been noted since; Hoffheinz (233) found that in 45 autopsies at which the parathyroids were found to be enlarged, osteitis fibrosa had been diagnosed 17 times, and osteomalacia 8 times. Erdheim (149) considered that these enlargements represented a vain attempt on the part of the parathyroids to compensate for some pathological condition which was deranging calcium metabolism, or as anal-

ogous to simple goiter. In accordance with this view, Mandl (334) treated a patient suffering from generalized osteitis fibrosa first with parathyroid tablets by mouth, and subsequently by transplanting four human parathyroids into the abdominal wall. As this treatment failed to improve the clinical condition, and the serum calcium was abnormally high, he adopted in place of Erdheim's a rival theory, and found and removed a tumor of one of the parathyroid glands; there was almost immediate clinical improvement and disappearance of the severe pains previously felt in the pelvis and lower limbs; the urinary calcium, previously much above normal, fell to a low level, and the serum calcium also decreased. Three years later (335) the patient, who had been completely bedridden, was able to work, but an increased density of the bones could not be perceived. Very similar cases were reported shortly afterwards by Gold (168, 169) and Barrenscheen and Gold (22). Another case was found and studied by DuBois and by Aub (203, 27, 418, 347); on this occasion no parathyroid tumor was found, and the operation was less convincingly successful. Very complete metabolic studies were made, and it was found that only on an extremely high calcium intake could the patient be maintained in calcium equilibrium; his condition was likened by Aub to that of a normal adult receiving 100 units of parathyroid hormone daily. Two cases were later reported by Barr (20, 21, 77), who suggested the introduction of the term "hyperparathyroidism," and one each by Boyd and Stearns (58, 478), Wilder (537, 533, 538), Eggers (335), Beck (32), Lanz (302), Snapper (470, 471), Hunter (255), Compere (113), Pemberton and Geddie (398), and Quick and Hunsberger (8, 408), and others, about twenty-nine in all (255). In addition to these we may note that several cases, certainly of this type, have been described in the literature but were not operated on (57, 138, 248, 129, 298) and no doubt there are many more. In fact it is now doubtful whether all cases of the generalized osteitis fibrosa first described by von Recklinghausen are not due to hyperactivity, with or without tumor, of the parathyroid glands, and some proportion of the cases diagnosed as osteomalacia (505) no doubt fall into this category likewise. The more common form of osteomalacia, however, is undoubtedly due to diet deficient in calcium and in vitamin D (344, 356), and is probably distinguishable by the low serum calcium. As Hoyle (245) has pointed out in an excellent review, Paget's osteitis deformans has not been shown to be related to hyperparathyroidism, and is therefore quite distinct from osteitis fibrosa generalisata.

A survey of these cases reveals a number of features of general physio-

logical interest. In spite of the fact that the serum calcium, before operation, is high, in one case (470) for a long time over 20 mgm. per cent, there is no sign, except in one dubious case (56), of the thickening of the blood, the congestion of the viscera, and the other symptoms which appear rapidly when the serum calcium of the dog is brought to this level. Closer examination reveals a further significant difference, in that the plasma inorganic phosphate in these clinical cases is normal or even definitely below normal (537). In nearly all cases, the amount of calcium excreted in the urine is abnormally high, but high fecal calcium has only been observed once or twice (298, 21, 255). The bones are decalcified to an extraordinary degree, so that pitiful deformity results (255, 408). After operation, the serum calcium in nearly all cases falls rapidly to a level much below normal, so that vigorous measures are required for a time to treat this new emergency; one case actually died in tetany (32). This suggests that the activity of the normal parathyroid glands is temporarily depressed by the hyperactivity of the enlarged one; it has similarly been observed in normal persons after prolonged treatment with parathyroid extract, yet with serum calcium normal or below normal, that withdrawal of the supply of hormone leads to a sudden temporary fall to still lower levels (4), as if the patient's own parathyroid glands had become temporarily inert. Experiments on dogs (240, 54), published since these words were written, have greatly strengthened the evidence for this view. In the same way the sudden fall of urine calcium to extraordinarily low levels, after removal of a parathyroid tumour, and the slow return to normal, can be imitated in normal individuals when a long course of treatment with parathyroid extracts is suddenly broken off. The calcium balance is much improved after removal of the tumor, in almost all cases; and sometimes the x-ray shadows of the bones have slowly become definitely denser, while in other cases this has not been seen. It would take us too far afield to discuss the pathology of the tumors found, which are usually adenomas; the literature since Langhan's classical description (301) has been briefly reviewed by Toland (506).

A group of Russian surgeons has formed the habit of removing part of the parathyroid tissue in cases of arthritis (477, 385), but it must be regarded as doubtful whether this procedure is justifiable, though high serum calcium is occasionally noted (338). In a series of fourteen cases, the serum calcium was above normal only five times (241). We may note incidentally, as a curiosity, that lowering of the serum calcium and clinical improvement in osteitis fibrosa have been reported to follow the

implanting of additional parathyroids into human subjects (402) and swine (508).

Before closing this discussion of the effects of excessive parathyroid hormone, exogenous or endogenous, in the body, we may discuss the relation of calcium to phosphorus once more. It has seemed to some that the effect of the parathyroid hormone may in the first place be upon the latter rather than the former (4, 151). Thus Greenwald has pointed out that after parathyroidectomy the decline in phosphorus excretion is more striking than the decline in calcium excretion (181), and that in parathyroid overdosage the extra excretion of phosphorus may occasionally be greater than that to be expected from the calcium and nitrogen excretion (185)—that is, the extra phosphate is not wholly derived from $Ca_3(PO_4)_2$ and from protein. Aub and his collaborators have made extended use of the "theoretical" phosphorus balances calculated in this way in their most careful studies, and they find that during prolonged treatment with parathyroid extracts there is good agreement with the balances actually found, except that when the treatment begins the excretion of phosphorus increases more rapidly, and decreases more rapidly when the treatment ceases, than is to be expected. Nor can this be ascribed to a lag in calcium excretion on the part of the kidney, since this is not seen when the excretion of calcium and phosphorus is increased by administration of thyroid or of ammonium chloride (4). This suggests that the sequence of events when parathyroid hormone is administered may be: phosphate excretion increases—lowers plasma phosphate—permits serum calcium to rise—increases calcium excretion. But it does not seem to us that this view is borne out by the data of an experiment in which urine and blood samples were taken hour by hour (5), nor by experiments on dogs (185), and certainly not by experiments on rats (407) in which a great increase in calcium excretion was not accompanied by any corresponding increase in phosphate excretion. Moreover, intravenous injection of parathyroid extract in dogs may raise *both* serum calcium and serum inorganic phosphate, while the latter may be enormously reduced by the administration of glucose and insulin without provoking any significant rise in serum calcium.

If we suppose the ratio of ionized serum calcium to total calcium to approach constancy, and further that PO_4''' ions are at any given pH a constant small fraction of the total inorganic phosphate, and finally that the relation $[Ca^{\cdot\cdot}]^3 \times [PO_4'''']^2 = K$ holds—this being the only logical means of correlating calcium and phosphorus—then rough calculation shows that decrease of inorganic phosphate from 4 to 2 mgm.

per cent permits a rise of calcium only from 10 to 16 mgm. per cent. It seems to us that the observed changes in total phosphate in parathyroid overdosage, in clinical hyperparathyroidism and especially in parathyroid tetany, are inadequate to explain the observed changes in calcium on this theoretical basis. If the various calcium fractions, discussed hereafter, do not vary together, or if the serum does not behave as if in equilibrium with solid calcium phosphate, then the effects of phosphate changes upon calcium must be still smaller—as, indeed, they are when glucose and insulin are administered.

The question deserves further study; but until reliable means can be devised for measuring the concentrations of $Ca^{\cdot\cdot}$ ions and $PO_4^{\prime\prime\prime}$ ions in the plasma, it will be hard to arrange a crucial experiment. It should not be forgotten that phosphorus exists in the body in very diverse combinations, many of them exceedingly labile.

The diuretic action of parathyroid hormone, repeatedly observed in normal human subjects (4, 5) and in dogs (251, 495) has prompted its use in nephrosis, and clinical improvement has been noted several times (127, 346, 340). The low serum calcium is not easily elevated, nor is the urinary excretion of calcium increased (312, 448).

The physical state of the blood calcium. It is now very generally agreed that the calcium content of the blood corpuscles is negligibly small; values around 0.3 mgm. per 100 cc. of cells are obtained by most recent workers, in several species (369, 483, 307, 473) and the higher values previously accepted may be ascribed to analytical errors. The figure quoted probably signifies that the interior of the corpuscles is practically free from calcium.

The relation between plasma calcium and serum calcium is not so clear. One might expect the serum to contain less calcium than the plasma, since fibrin contains calcium (357, 323)—more, indeed, than does a precipitate of fibrinogen (119)—much of which can be removed by thorough washing. In practice, however, the anticoagulant draws water from the cells, so that plasma is more dilute than serum, and has accordingly a lower concentration of calcium and other constituents (82, 369, 323, 483). These effects may cancel out; thus Stewart and Percival (483) found that where serum and citrate plasma both contained 10 mgm. per cent, true plasma obtained without the use of an anticoagulant had 12 mgm. per cent of calcium. Cameron and Moorhouse (82), allowing for the effect of the anticoagulant, found that serum calcium and plasma calcium were normally equal; they considered, however, that much of the plasma calcium was present in a form which

reacted only slowly with oxalate, but which was somehow changed during clotting so that four-fifths of the serum calcium may be precipitated in a few minutes. The rôle of calcium in the clotting of the blood is a question which lies outside the scope of this review; be it noted merely that the amount of oxalate or fluoride required to prevent clotting is three or four times the equivalent of all the calcium present (519, 484, 323, 369). Loucks and Scott (323) have found that about 6 per cent of the total serum calcium may be extracted with ether; yet at present it is probably superfluous to postulate the existence of specific ether-soluble compounds, such as the calcium phosphatidate (93) found in plants.

We come now to an extremely thorny problem. It has been generally recognized since the work of Rona and Takahashi (431) that if serum be dialyzed against inorganic solutions of various calcium concentration, the fluid with which no shift of calcium is observed contains only 5 or 6 mgm. of calcium (436, 354, 118, 517). Similarly, an ultrafiltrate of serum contains only half of the total calcium, or slightly less (374, 458), if the filtration be carried out at a suitable pressure. It has thus become customary to divide the total serum calcium into two fractions, usually referred to as *diffusible* and *non-diffusible*. The question now arises, in what form is the non-diffusible calcium? Since the relation is not disturbed by extracting the serum with ether, lipoid-calcium combinations may perhaps be excluded (321), though we may point out that a large lipoid fraction remains in ether-extracted serum. To Cameron and Moorhouse (82), for reasons to be discussed presently, it seems that the non-diffusible calcium is a specific compound whose formation is controlled by the parathyroid hormone. Most workers hold, however, that the non-diffusible calcium is bound to the various serum proteins.

Thus it has been shown that when the serum proteins are precipitated each fraction carries with it more or less calcium (119). The apparent solubility product of calcium carbonate is much increased by addition of purified serum albumin, to a less extent also of serum pseudoglobulin; as this effect is much less marked with calcium sulphate, Pauli concludes that calcium proteinates are formed (395, 397). But the observation that albumin may bind as much of both calcium and potassium from mixtures as from solutions containing only one of these cations (166) is not easily reconciled with so simple a view. Purified serum proteins tend to retain added calcium in dialysis experiments, to an extent not to be explained by invoking Donnan effects (319, 339). The addition of calcium chloride to serum made almost free of electrolytes by dialysis

increases the conductivity less than it should if the added ions remained free to transport the current (457), just as purified proteins decrease the conductivity of zinc chloride solutions (396); the magnitude of these effects seems to be too great to be explained merely as depression of the activity of the added ions by the electrostatic fields surrounding the protein ions.

It has been shown that the apparent solubility product of calcium phosphate in diluted sera displays a minimum at a pH near the iso-electric point of the proteins (120), and it is said that the non-diffusible calcium of the serum vanishes as the iso-electric point is approached (435, 282), but others have not found it to vary with CO_2 tension. The amount of the non-diffusible calcium in various species runs roughly parallel to the average serum-protein content (517). In nephrosis, low serum protein and low serum calcium exist together (441), and it is said to be the non-diffusible fraction which is diminished (317); on the other hand, when serum protein was greatly reduced, in dogs, by repeated plasmapheresis, there was little change in calcium, but a marked increase in inorganic phosphate, so that a sample of serum contained, for example, 0.82 per cent albumin, 3.53 per cent globulin, 10.65 mgm. per cent calcium, and 12 mgm. per cent inorganic phosphate (19). Protein-poor serum has lost much of its power to dissolve unusually large amounts of calcium carbonate (210).

Summing up, we feel that a very large body of evidence has been presented to show that a large part of the serum calcium is less diffusible than the remainder, and that this non-diffusible fraction is some combination of calcium with protein, but at the same time that if the evidence be taken piecemeal a sceptic might offer much damaging criticism. Plausible calculations show that the Donnan membrane effects can explain only a small part of the anomalous behaviour of the calcium in compensation dialysis of serum (179). It is clear that the non-diffusible calcium can very readily become diffusible: for example, though it requires M/40 oxalate to prevent coagulation of the blood, M/320 oxalate will precipitate 90 per cent of the serum calcium in seven minutes (369); again, if serum be dialyzed, not against calcium-containing solutions, but against 0.6 per cent NaCl, it rapidly becomes almost free from calcium (318, 320). We have already quoted evidence to show that added inorganic calcium may in part come to behave as non-diffusible (319, 457, 339). The conclusion appears to be that there is a dynamic equilibrium between the two calcium fractions, and the evidence against this view (517) must be interpreted in some other way.

It seems to be rather likely that the amount of non-diffusible calcium in any sample of serum depends upon the total amount of calcium, the amount of serum protein, and the pH (320, 339).

It has usually been concluded that the sodium and potassium, and possibly also the magnesium, are completely diffusible. This view is not supported by the recent work of Greene and Power (178), who devised an ingenious method of *in vivo* dialysis, in which the volume of dialysate is so small relatively that the composition of the plasma cannot be significantly affected. They found chlorides to be more concentrated in the dialysate than in the plasma, and took this difference as a measure of the magnitude of the Donnan membrane effects produced by the inability of the proteins to dialyse, obtaining the coefficient $r = 0.9809$. The cations were naturally more concentrated in the plasma than in the dialysate (concentration being expressed in terms of milliequivalents per kilogram of water), but this discrepancy was greater than the former one, and could only partly be explained as due to the Donnan effect. Assuming that the excess of non-diffusible base was present in the form of unionized base-protein compounds, they find that 7.4 per cent of the total sodium, 20.4 per cent of the potassium, 39.7 per cent of the calcium, and 29.1 per cent of the magnesium was present in this undissociated form; so that the behaviour of calcium is quantitatively rather than qualitatively remarkable. We wish to make four comments on these most interesting results. The first is that the difference between activity and concentration must be greater in plasma than in the dialysate, since the proteins add to the ionic strength; it may be that this difference affects the cations more than the chloride ions, and that the apparently excessively high concentration of base in the plasma does not represent an excessively high activity. Secondly, if it be true that a considerable part of the calcium is present in plasma as an unionized diffusible compound (a point discussed below), then it appears that the degree of dissociation of the hypothetical calcium-protein compounds must be much less than the 10 per cent suggested by Greene and Power, who ignore this possibility. Thirdly, it has been shown by Hill (226) that the observed vapour pressure of blood can be almost exactly accounted for by summing the molar concentrations of its known constituents; sodium and potassium together make up about half of these, and if a fraction of these bases, of the order suggested by Greene and Power, is not ionized and cannot be taken into account, a discrepancy of 3 or 4 per cent appears between the observed and the computed vapour pressures; which means that constituents of the blood as signifi-

cant osmotically as the sugar *plus* the urea must still be searched for, unless the data can be reconciled in some other way. Fourthly, the assumption that the chloride distribution is a measure of the Donnan coefficient may be incorrect, since the recent work of Van Dyke and Hastings suggests that non-diffusible chloride fractions exist in the corpuscles at least, and hence their absence elsewhere can hardly be taken for granted.

Attempts have been made to measure by various direct methods the concentration of calcium ions in serum, or in its ultrafiltrates or dialysates. Neuhausen and Marshall (373) used a flowing calcium-amalgam electrode, and concluded that not more than 10–20 per cent of the total serum calcium was ionized. Corten (116) used a "third-order" electrode (zinc-zinc oxalate-calcium oxalate) and obtained similar values. But it does not appear to be established that such systems are capable of measuring calcium ion activities accurately even in simple inorganic solutions. Brinkman and van Dam (65) devised a method based on the idea that oxalate would react instantaneously with ionized calcium, and only slowly with calcium present in other forms in ultrafiltrates of serum; and they reached similar values. Though this method has been used several times, it has been very hotly criticized, for example on the ground that supersaturation of the serum with calcium oxalate cannot be ignored (446). A method of biological assay was proposed by Trendelenburg and Goebel (509), who found that the isolated frog heart responds to changes in the calcium ion activity of the perfusing medium by changes in amplitude of beat; this method has yielded the unexpected (and theoretically suspect) result, that addition of carbonate or phosphate to serum does not affect the amount of ionized calcium (134, 332).

Direct evidence that the whole of the diffusible calcium is not ionized is furnished by the finding that some of the calcium in serum ultrafiltrates (284), as in serum (42), does not travel to the cathode in electrodialysis. This problem, however, has more often been attacked from a theoretical angle, since Rona and Takahashi (433) applied to serum Bodländer's formula:

$$[Ca^{\cdot\cdot}] = K \frac{[H^{\cdot}]}{[HCO_3']}$$

This was later amplified by Warburg (525), who, taking account of the effect of the ionic strength of serum on calcium ion activity, concluded that 1.8 mgm. per cent represented the concentration of calcium ion in serum. But since it appears that phosphates have a specific

influence upon the solubility of calcium carbonate (339, 210) and oxalate (201), such arguments are not wholly convincing. Holt, LaMer and Chown (236) calculated the concentration of PO_4''' ions in serum from the total inorganic phosphate, the hydrogen-ion concentration, and the three dissociation constants of phosphoric acid. These figures lead to the result that about six parts per million of the total inorganic phosphorus are present as PO_4''' ions at the pH of serum. Now since the square of the PO_4''' ion activity, multiplied by the cube of the $Ca^{..}$ ion activity, must be equal to a constant (the solubility product), *if* the serum is in equilibrium with solid calcium phosphate, the extent to which the calcium is ionized could be computed. Since the apparent solubility product as found in serum was much greater than that obtainable in salt solutions, they concluded that serum was 200 per cent supersaturated with calcium phosphate; even in rickets it was supersaturated (237). They supported this by experiment, in that they shook serum with solid calcium phosphate, and finding the amount of soluble calcium greatly reduced concluded that the excess calcium phosphate had precipitated. But it was later found that in such experiments the loss of soluble calcium is not accompanied by a loss of phosphate, but rather it is calcium carbonate which precipitates on to the solid phosphate (451, 282, 284). The fact that the soluble calcium of the serum is not affected by freezing and thawing also speaks against supersaturation (284). It may be remarked in parenthesis that in our experience serum calcium determinations give low results when the serum or blood has been chilled. Hastings, Murray and Sendroy (210) made a careful study of the effects of shaking calcium carbonate with serum, as compared with salt solutions of similar ionic strength. They concluded that only a small part of the total calcium was present as ions, about 1.5 mgm. per cent, and that the excess was only partly accounted for as calcium protein complex; moreover, they believed that the presence in serum of significant amounts of dissolved undissociated $CaCO_3$, $CaHPO_4$, and $Ca_3(PO_4)_2$ could be excluded. When similar studies with calcium phosphate were made (451) an even smaller value for calcium ion concentration was obtained. But it must be observed that even if pure, solid $Ca_3(PO_4)_2$ is obtainable, which is by no means certain, it would decompose on shaking with aqueous media and form basic phosphates (322), so that experiments of this type are unreliable. Furthermore, the assumption is always made that the whole of the measured inorganic phosphate of the plasma is present either as undissociated phosphoric acid or as one or other of the three possible ions, and is all diffusible

(432, 403). But by *in vivo* dialysis Brull (74) found that this was not the case, and Greene and Power (178) found that about 15 per cent (calculated by the reviewers) of the phosphate did not distribute itself in accordance with the Donnan equilibrium, but remained in the plasma. Comparable results were obtained with ascitic fluids (179), and it is known that the cerebro-spinal fluid contains less than half of the inorganic phosphorus of the plasma (352, 387, 199). Eicholtz and Starling (142) believed that added inorganic phosphate rapidly passed into a colloidal state. For these reasons we are inclined to prefer the data obtained with calcium carbonate to those obtained with calcium phosphate, especially since the former are in agreement with the results obtained, however crudely, by direct measurement, and to estimate the concentration of ionized calcium in serum at 1.5 to 2.0 mgm. per cent. But we would point out that the assumption not infrequently made, that the concentration of calcium ions in an ultrafiltrate or dialysate of plasma or serum is the same as in the plasma itself, is not necessarily correct.

We then have to account for the presence in serum of some 3.5 mgm. per cent of calcium in an unionized, diffusible form. Greenwald (184) suggested that it was a compound similar to calcium citrate, quite slowly precipitated by oxalate or phosphate, in equilibrium with the ionized fraction, and in some way promoted or maintained by the parathyroid hormone. It is known that citrate decreases the conductivity of calcium salts (454, 455), and that in its presence the deposition of calcium on rachitic cartilage in inorganic calcium-containing solutions *in vitro* is inhibited (460). Even at such dilution as 0.0025 N calcium citrate is only 25 per cent ionized (540). But Hastings, Murray and Sendroy (210), studying the high solubility of $CaCO_3$ in citrate solutions, believed that if a calcium-citrate complex was formed it could not have the expected composition $Ca_3(C_6H_5O_7)_2$. Shelling and Maslow (458) find that injections of citrate decrease the non-diffusible calcium of the serum, the citrate apparently taking calcium from the protein. Then the anticoagulant reaction of citrate comes into line with the conception (323, 484) that it is not ionized calcium but some calcium complex which plays a vital part in the coagulation of the blood. But we must make it clear that very delicate methods for the detection of citrate are now available, and assure us that this puzzling diffusible calcium fraction probably is *not* a citrate, however similar it may be, the citrate content of human blood serum (503) being too small to carry the amount of calcium involved.

It is plausibly supposed that only the ionized calcium is of importance in connection with the excitability of nerve and muscle. This view is supported to some extent by experiments on isolated tissues and organs, such as those of Loeb. In accordance with it is the observation that in nephrosis and kala-azar, where the non-diffusible fraction is much reduced, presumably because of the low plasma protein content, tetany is not seen (317). We then have to consider, first the ionized calcium, which at constant pH will depend on the concentration of inorganic phosphate, *provided* that the serum behaves as if in equilibrium with solid $Ca_3(PO_4)_2$, and on CO_2 tension also, if it behaves as if in equilibrium with solid $CaCO_3$ (though in fact it seems that serum cannot be brought into equilibrium with added solid $CaCO_3$ (210)); secondly, an anomalous and puzzling undissociated diffusible fraction; thirdly, a non-diffusible fraction, which at constant pH depends partly at least upon the concentration of protein. This, it seems to us, is the view which best fits the large mass of experimental data above recorded, as well as the experiments on production and relief of tetany; and it is the view generally accepted, although, as we have already pointed out, no single one of the links in the connecting strands of evidence is entirely trustworthy. In accordance with this line of thought, Peters and Eiserson (399) have developed an empirical equation connecting serum calcium with protein and with inorganic phosphate, expressing Ca and P in milligrams per cent, and protein in grams:

$$\text{Ca} = -0.255\ \text{P} + 0.566\ \text{protein} + 7$$

and believe that departure of observed calcium values from those calculated in this way indicates some disorder of *calcium* metabolism itself. Such an equation may be useful practically, but can have no theoretical significance. Greenwald (188) has discussed the question and proposed alternatives. It is remarkable, in fact, how closely the inverse relationship between total calcium and total inorganic phosphate in serum may appear to obey the law of mass action in its simplest form, so that the product Ca $\times$ P may become constant at very various levels of parathyroid function, both in hyperactivity and hypoactivity (6). On the other hand, it must be emphasized that in acute experiments, for example after intravenous injection of calcium solutions, the observed fluctuations in serum inorganic phosphate are usually quite small and not always in the direction anticipated. It is obvious that there is much that is obscure in the whole question, especially as regards the inter-relation between the different calcium fractions.

The effect of parathyroidectomy and parathyroid administration on the calcium partition. Cameron and Moorhouse (82) believed that while serum calcium and plasma calcium were normally equal, after parathyroidectomy a considerable amount of calcium was retained by the clot; this has not been confirmed (183, 369). The suggestion (18) that the amount of calcium in the corpuscles increased in tetany has been denied (369), but recently revived by Sokolovitch (473), who apparently has overlooked this previous work. It may be observed that his data, consisting as they do of determinations of serum calcium and whole blood calcium, would fit the theory of Cameron and Moorhouse quite as well as they do his own.

Parathyroidectomy does not decrease serum protein (442) nor does it increase hydrogen ion concentration; we may therefore assume that if the non-diffusible calcium is diminished, it is because some other calcium fraction is being drawn on and is disturbing the balance between the fractions. The ratio of non-diffusible to diffusible might then be expected to increase, at any rate at first, and claims of this sort have been made (403, 411). But the majority of investigators have found the ratio in tetany to be either unchanged (355, 370, 369) or decreased (118, 82, 214). If it be true that the undissociated diffusible fraction is closely dependent on parathyroid function, we might expect that the administration of the hormone should lead primarily to an increase in that fraction, especially since plasma protein does not increase (317). That the ratio of non-diffusible to diffusible does decrease has been reported several times (520, 482, 317); but it has also been said to increase (214) or to show no change (320). Grollman also believes that parathyroid overdosage reduces the diffusibility of the inorganic phosphate, and that a similar reduction can be obtained by adding calcium chloride to the serum *in vitro*. Parathyroid extracts, added to serum *in vitro*, increase the apparent solubility of calcium phosphate (451), but there is no reason to believe that any other similar proteose would not have similar effects.

It has long been a popular idea that the cerebrospinal fluid calcium represents the diffusible calcium of the plasma more accurately than any artificial dialysate or ultrafiltrate can, although it is not clear that the cerebrospinal fluid is not actively secreted by the choroid plexus; this is a controversial subject into which we cannot enter here. Normally, the cerebrospinal fluid contains about 5 mgm. per cent of calcium, that is to say, half as much as serum (87, 82, 369, 387, 352), and in general less than the diffusible calcium obtained from serum experi-

mentally (517, 176). When dialyzed against serum, it appears to gain in calcium (369). After parathyroidectomy the level of calcium in the cerebrospinal fluid may decrease, but much more slowly than the serum calcium, so that the two may approach equality (82, 214, 369, 353). The administration of parathyroid extracts is generally conceded to have but little effect on cerebrospinal fluid calcium, or at any rate the increase is slow (83, 369, 353). This is contrary to the findings of Berencsy (36), but his control values are much higher than those of other investigators. Very recently it has been stated that the cerebrospinal calcium rises rather rapidly to a peak in three or four hours, and returns to normal before the serum calcium has attained its maximum (88, 353). One might venture to interpret this as an indication that the first action of parathyroid extracts is to increase diffusible calcium, but that a rearrangement slowly follows until all fractions are elevated. In nephrosis, with low serum protein, it is difficult to raise the serum calcium with parathyroid extracts. Several of the investigators who have studied the behaviour of the cerebrospinal fluid calcium have used in interpretation the idea that it comes only slowly into equilibrium with the serum calcium, and that its apparent constancy is mere inertia. Thus in their careful study of the subject, Morgulis and Perley (369) found that intravenous injections of calcium-containing Ringer's solution, sufficient to raise serum calcium by 5–18 mgm. per cent, raised cerebrospinal fluid calcium only 0.7–2.7 mgm. per cent, and this relation was maintained after parathyroidectomy. Although this idea does not seem far-fetched, there may be cited against it not only the above-mentioned *rapid* changes (88, 353), but also a recent experiment by Merritt and Bauer, who drained off the cerebrospinal fluid for a long period, during which much water was given by mouth and diuresis was prevented by the use of pituitrin; even when there was a total decrease of some 5 per cent in serum calcium, phosphate, and protein, the *ratio* of cerebrospinal fluid calcium to serum calcium remained unchanged at 0.5 (352).

The thoracic duct lymph (44), the aqueous humor (352), ascitic fluid (179), and amniotic fluid (352) all contain more calcium than cerebrospinal fluid, but less than serum. Nor is it possible to relate this to the protein content of these fluids, since amniotic fluid, for example, contains only 0.2 per cent protein; yet it has often been noted that the ratio of cerebrospinal fluid calcium to serum calcium is high when the fluid contains abnormal amounts of protein. It appears to us that these discrepancies should be regarded as a warning against the facile assump-

tion that all such transudates may be immediately compared to dialysates of whatever sort. It has been noted that the calcium content of the aqueous humor, in rabbits, is only slightly raised by parathyroid administration (353).

The view that one or other of the unionized calcium fractions owes its existence to the presence of the parathyroid hormone implies that the ratio of ionized to total calcium tends to be constant; otherwise, how could a decrease in an undissociated compound precipitate tetany? The argument we have previously advanced, that on the basis of the equation

$$[Ca^{\cdot\cdot}]^3 \times [PO_4^{\prime\prime\prime}]^2 = k$$

the changes in phosphate at various levels of parathyroid function are not commensurate with the observed changes in calcium, may be cited as evidence that some unionized calcium fraction *is* controlled by the parathyroid, and hence that the ratio of ionized to total calcium must somewhat increase after parathyroidectomy and decrease in hyperparathyroidism. We see no difficulty in supposing that parathyroidectomy leads to a decrease in some special undissociated calcium compound, this in turn to a decrease, relatively less but sufficient to cause tetany, of the ionized calcium (of which the phosphate may take advantage to increase). Nor does it seem impossible to suppose that the ratio of ionized calcium to what may be termed the "parathyroid fraction," already raised after parathyroidectomy, may be still further raised with relief of tetany by the administration of calcium salts (no doubt with decrease of phosphate). This might also involve a rise in a calcium fraction not ionized but not directly dependent on the parathyroids. On the other hand, such a hypothesis must not be put forward too simply. We believe that the evidence is against the idea that a calcium compound of the parathyroid hormone circulates in the blood serum. The serum of parathyroidectomized dogs does not show any failure to dissolve calcium salts *in vitro* (210). When calcium chloride is injected intravenously, and parathyroid hormone subcutaneously at the same time, the rise in serum calcium due to the injected calcium, has completely subsided before the rise due to the parathyroid extract is appreciable (110). It might be objected that absorption from the site of subcutaneous injection may be slow. Experiments have therefore recently been carried out in which calcium chloride solution and parathyroid extract have been given simultaneously intravenously to dogs. The results are perfectly definite; the serum calcium, high imme-

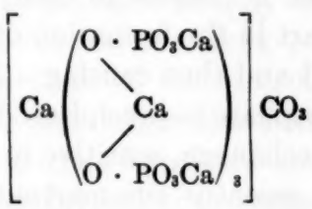
diately after the injection, falls rapidly during the first hour or more; the subsequent rise due to the parathyroid extract is appreciable two hours after injection and rises steadily to a maximum. The resulting double-peaked curve of serum calcium values does not suggest any interaction between the parathyroid extract and the injected calcium. The fluctuations in serum inorganic phosphate are irregular and insignificant. Similar results were obtained in rabbits by Inaba (257). These experiments appear to lead to one of the three following conclusions: a circulating calcium compound of parathyroid hormone *is* formed, but very slowly even in the presence of excess calcium; the parathyroid hormone stimulates the formation from an unknown source of an unknown calcium-binding substance; or finally, the parathyroid hormone acts primarily on the bones, causing liberation of calcium by some direct stimulating action, rather than by increasing the solvent power of the serum for the calcium compounds of bone. Of these alternatives, we believe the third to be the simplest and most attractive.[4] Its adoption, however, necessitates the further assumption that the serum is not normally saturated with respect to the solid calcium compound or compounds of bone. It appears to be unlikely that the blood serum itself is in contact with the mineral substance of bone; and nothing relevant is known of the equilibria which connect the serum with such tissue fluids as actually are in contact with this solid phase. The only evidence in favour of the view that serum is saturated with respect to the calcium compounds of bone is furnished first, by the experiments on calcification *in vitro* (to be discussed later), in which the mechanism involved is probably quite complex, and secondly, the vague general tendency of serum calcium and serum inorganic phosphate to vary inversely in experiments sufficiently prolonged. But these findings can only be cited in this argument if it is assumed that the bone mineral dissociates to yield calcium ions and some species of phosphate ions. We regard this assumption as plausible, although there is much divergence of opinion as to the composition of bone.

The relation of the parathyroid hormone to the calcium of the skeleton.

[4] It is also in accord with the histological evidence. The studies of Bodansky and Jaffé (52) and of Bulbring (1931, Arch. exp. Path. u. Pharm., clxii, 209) suggest active rather than passive liberation of calcium from the bone. Still more clearly, H. Selye (1932, Arch. Pathol., in press) has shown that parathyroid extracts lead to formation of osteoclasts from fibrous connective tissue, and that in very young rats subcutaneous fibrous tissue may also proliferate, producing an experimental scleroderma.

The difficulties of separating salts which, though of low solubility, readily undergo hydrolytic decomposition, are so great that even today little is known of the composition of bone except the relative amounts of the various elements present; there is hardly any definite information as to the manner in which these are combined. Two possibilities must be considered; first, that the mineral material is a mechanical mixture of different solid phases, each of which may have a quite simple composition, as $CaCO_3$, $Ca_3(PO_4)_2$, and the like; and secondly, that the greater part of the mineral material consists of crystals which are of constant composition, except in so far as replacement of one group by another, as carbonate by fluoride or hydroxyl, may take place without disturbing the crystal lattices, by isomorphism. Clearly, this dilemma has a close bearing on the question whether serum may be regarded as behaving as if in equilibrium with solid $Ca_3(PO_4)_2$ or not. In sympathy with the first view may be cited Howland, Marriott and Kramer (244), who affirmed that, if one supposed all the carbonate to be $CaCO_3$, and all the magnesium to be $Mg_3(PO_4)_2$, the ratio of residual calcium to residual phosphorus was 1.94:1, as in $Ca_3(PO_4)_2$. But to this it may be rejoined that later determinations have found this ratio to be in fact somewhat higher, especially in newly-formed bone (453, 290); that $Ca_3(PO_4)_2$ is not a known mineral, and is unstable in contact with water, losing phosphoric acid and forming a basic phosphate (322); and that magnesium carbonate is quite probably present (283). The results of crystallographic analysis by x-ray are much more in favour of the second view (269, 499, 438, 95). Taylor and Sheard (499) concluded that in physical properties such as x-ray diffraction and refractive index bone approached more nearly to podolite, $CaCO_3 \cdot 3Ca_3(PO_4)_2$, than to any other known mineral, though in fact the fit is not strikingly good; they believed that the presence of $CaHPO_4$ could be excluded. Recently Roseberry, Hastings and Morse (438) by x-ray spectrographic analysis found that both $CaHPO_4$ and $CaCO_3$ (as calcite) could be excluded. They believe that $Ca_3(PO_4)_2$ may exist in crystalline form but did not demonstrate its presence in bone, which, they believe, approximates in composition to podolite, or to dahlite, $CaCO_3 \cdot 2Ca_3(PO_4)_2$. Such formulae, it seems, may sufficiently define the nature of a homogeneous crystalline solid, which on contact with water may be expected to yield the same ions, $Ca^{\cdot\cdot}$, CO_3'' and PO_4''', as a mere mechanical mixture of the two solid phases. Others, however, have followed the lead given many years ago by Werner and prefer to rewrite the formula as a complex salt. Gassmann (164) warmly champions this idea, and believes

that he has been able to obtain the chloride of a complex cation in solution; but the demonstration is not convincing. On such a view the composition of bone is represented by



or some similar structure. On any theory of this type it is obvious that phosphate cannot be set free from bone without simultaneously dislodging the carbonate and *vice versa*. Further, it would seem that on this basis the $Ca_3(PO_4)_2$ solubility product should be irrelevant to calcification, and perhaps that complex calcium-phosphate cations should circulate in the serum. Klement (283) believes that $CaHPO_4 \cdot 2H_2O$ is first deposited but undergoes hydrolysis to form a semi-basic phosphate of the composition $6Ca_3(PO_4)_2 \cdot Ca(OH)_2$, with which some $CaCO_3$, forming a second solid phase, is intermingled. He believes that magnesium and other bases, and a fraction of the carbonate, are not present in the solid phase in living bone.

It has been found that ossifying cartilage, taken from rachitic rats, will form solid calcium deposits when immersed in healthy serum (459) or in salt solutions of similar calcium and phosphorus content (460), though, as Robison pointed out, the two cases are not comparable, since the salt solutions lack the protein and whatever specific calcium-carrying groups there may be in serum, and are therefore certainly supersaturated. None the less this work does indicate that blood composition is a limiting factor in calcification and of the highest significance in rickets. It is worth pointing out, since misunderstandings have frequently arisen, that Shipley and Holt (460) said that "the process is clearly not one of simple precipitation; it depends upon the activity of living tissue." The recalcification occurs only when the product Ca $\times$ P in serum or salt solution (in milligrams per cent) is greater than 30; of course this product is purely empirical, since it includes all fractions of calcium and of phosphate. A sufficiently high pH is necessary (460, 289) and a low Ca:P ratio is unfavorable (426). It has been contended that a low blood phosphate is not always met with in active rickets (341, 425, 220), and that irradiated ergosterol may raise blood phosphate yet fail to establish healing (220). This latter point has been hotly con-

tested, but it seems to us that different interpretations of what constitutes healing are largely responsible for the controversy.

Robison, Kay, and their colleagues have made very thorough studies of the phosphatase which is present in ossifying cartilage, and which they believe to play a part in the formation of bone, by breaking down esters of phosphoric acid and thus causing a local excess of phosphate ions so that calcium phosphate is precipitated. It is agreed, however, that some other active mechanism, sensitive to cyanides, is also involved (426). Kay (277) has recently summarized the evidence that the enzyme plays a part in ossification; it hydrolyses the soluble calcium salts of many esters of phosphoric acid, so that in alkaline solution calcium phosphate is precipitated; it is present especially in growing bone, and is absent from cartilage not destined to undergo ossification; rachitic bone will calcify in glycerophosphate or hexosephosphate solutions, and is by no means deficient in enzyme (425, 197) and subcutaneous injections of calcium glycerophosphate may promote healing in rachitic rats (288); a substrate for the enzyme is normally present in the blood. Robison has shown that in the calcification of rachitic cartilage in artificial solutions, the inorganic phosphate may be largely replaced by organic phosphate (426), and that explants of femur from six-day chick embryos showed ossification and phosphatase activity on cultivation *in vitro*, while the non-ossifying part of Meckel's cartilage did not (156). In diseases of bone the phosphatase content of the blood plasma, ordinarily quite small, is much increased; for example, in rickets, osteitis deformans, and osteitis fibrosa (276, 277, 423). After injections of parathyroid hormone, the bone phosphatase is decreased (389, 390, 224, 498), plasma phosphatase has been seen to increase (390, 53) in some cases and to decrease (53, 498) in others; in experimental osteitis fibrosa in guinea pigs it is high (53). Jenner and Kay (261) have recently reported that over a certain pH range magnesium accelerates both the hydrolytic and the synthetic action of phosphatase. Magnesium inhibits the recalcification of rachitic cartilage *in vitro* (461, 462, 289). Rats reared on a diet of very low magnesium content show an extreme decalcification of the skeleton (348). We place these facts together without comment.

Hypertrophy of the parathyroids has been noted in rickets in man (422, 391), in rats (149, 450, 328), and in fowls (336, 225, 379). Erdheim's view was that this represented an attempt to compensate for the disorders in calcium metabolism imposed, as we now know, by a deficient diet. The later workers find that hypertrophy of individual

cells is the outstanding feature, and that eventually (379) there is a regression of the gland. Rickets can be produced in parathyroidectomized rats as readily as in normal rats (286, 392). Bischoff (48) believes that parathyroid hormone treatment may serve to prevent rickets in puppies, and, as we have already said, Bauer, Aub and Albright found that in rats treated over long periods the bones, though shorter, were denser than those of the controls (25), and an increased percentage of calcium in the bones has been noted in fowls (498). Hueper (250) also believes that formation and calcification of osteoid tissue is increased in parathyroid overdosage. But the influence of parathyroid treatment has been found to be unfavourable in rickets in rats (524) and children (231).

The healing of fractures appears to depend on the same set of conditions as the healing of rickets, notably the calcium and phosphorus content of the plasma (213). During healing, the serum calcium and especially the inorganic phosphate may be elevated (124). The bad effect of parathyroidectomy on the healing of rickets is most simply explained as the result of the low calcium (135, 439). It is not so easy to understand why injections of parathyroid (157, 306) or of calcium salts (381) should retard healing. Yet in hyperparathyroidism, with its frequent spontaneous fractures, callus formation is not impaired (408).

A question of the greatest interest is that of the reversibility of calcification. Gamble, Ross and Tisdall (163) noted that in fasting, with ketosis, they could account for the loss of sodium, potassium and magnesium by allowing for loss of extracellular and intracellular water, with its dissolved salts, but that the urinary excretion of calcium was ten times greater than would be expected on this basis; the excretion of phosphorus was too great to be wholly explained by breakdown of protein, but not so great that they felt it necessary to regard it as originating in the bones. Goto (171) believed that in the increased calcium and phosphorus excretion produced by administering hydrochloric acid, the calcium was derived from the bones but the phosphorus from tissue phosphate; but the analyses on which this surprising conclusion is based are unconvincing, having been performed on bone that was not defatted. Irving (259) found that the amount of CO_2 given off by eviscerated cats in overventilation, or retained by them in breathing CO_2-rich mixtures, was too great to be accounted for by changes in the blood and muscle; though the amounts involved, some 300 cc. per kilo, were too small to be detected in bone analyses. The idea that the bones may act as an additional alkali reserve is similar to that put forward

by one of us (98) for the shell of molluscs. It is obvious that in discussing the work of Goto, and of Irving, it is necessary to be clear which theory of the composition of bone is being kept in mind. The work of the Boston group (26) is in accordance with McCrudden's conception (349) that there is a constant "wear and tear" of the bones, an endogenous metabolism of calcium, which, once liberated, cannot be used again, even while bone-building is actively going on. Aub and his colleagues (15, 153) have fully discussed the possible rôle of calcium in the neutralization of acid. Bauer, Aub and Albright brought forth very good evidence that the trabeculae of the bones form a mobile store of calcium. They showed that they were depleted by parathyroid administration (25) in the rabbit, as in the rat (299). They also placed cats upon a calcium-rich diet for many weeks, amputated one limb, and then continued the experiment with a calcium-poor diet, and found that in the latter half the trabeculae were much reduced; the converse experiment showed an increase in the trabeculae on a calcium-rich diet (25). This agreed with the finding of Hunter and Aub (253) that in lead poisoning the administration of parathyroid extracts led to the excretion of both lead and calcium; if after a rest period the treatment was recommenced, calcium alone was excreted; this pointed to the existence of a mobile store of calcium, originally contaminated with lead, but readily replaced after depletion by pure calcium salts. Alizarin red is known to stain only newly-deposited bone; by its use it was possible to show that when the calcium intake was increased new trabeculae were formed (25).

Morgan and Garrison (367, 368) have found that in young dogs reared on diets deficient in calcium, or in vitamin D, it is difficult to raise the serum calcium with parathyroid extracts; this suggests the absence of a store of calcium upon which the hormone has a solvent action. Bodansky and Jaffe (52) are led by their experiments on the action of the hormone over long periods to challenge Aub's view of the lability of the trabeculae, and believe that the bone most recently laid down, in whatever site, is the bone most readily redissolved. György (194) believes that the calcium of the sub-epiphyseal bone layers is most readily mobilized.

We believe that the possibility of calcium salts being transferred from one part of the bone to another has not been sufficiently considered; few experiments have been planned to take account of this. Hamilton (198) produced evidence to show that the fetus, just before birth, accumulates a store of calcium which is used in bone growth just after birth. In very young kittens the trabeculae are reduced and

alizarin shows that new deposition on the corticalis is taking place (25). Morelle (366) has demonstrated shifts of calcium within the bone in rickets, and they appear to occur also in overdosage with irradiated ergosterol, where parts of the bone are hypercalcified and others rarefied (206).

Vitamin D in relation of the parathyroid glands. Since vitamin D certainly resembles the parathyroid hormone at least inasmuch as it tends to raise or to maintain the level of the serum calcium, it is desirable to scrutinize the relation existing between them. In experimental rickets in rats, which is commonly produced by a low-phosphorus high-calcium diet, the composition of the blood somewhat reflects that of the diet, the excess of calcium fed tending, no doubt, to precipitate phosphate in the intestine. The addition of vitamin D causes a rise in organic phosphate and sometimes a fall in calcium in the serum, so that, as we have seen, signs of tetany may appear. In this case, we must suppose that the absorption of phosphate has been improved; but there is equally good evidence that when calcium deficiency is in question, as so often in osteomalacia (344), the vitamin may improve calcium absorption. This has been shown to be true even of excessive doses of irradiated ergosterol, up to the point when severe toxic symptoms supervene (209). The mechanism of this action is unknown. Calcium absorption depends to some extent upon intestinal pH (542, 386), no doubt because alkalinity favours precipitation of the phosphate by increasing PO_4''' ion concentration; for the same reason, an excess of phosphate also lessens calcium absorption (386). It has frequently been remarked that in rickets the contents of the colon and the feces are alkaline, whereas they are normally acid; and that vitamin D, in curing the rickets, restores the normal reaction (543, 409, 1, 262, 174). It seems to us that this change may as well be the *result* as the *cause* of the improved absorption of base, since there is little reason to suppose that minute amounts of the vitamin could have any acid-producing action; in fact, the evidence suggests that on the contrary it reduces hydrochloric acid secretion and makes the pancreatic secretion more alkaline (28), and it is impossible to produce abnormal fecal acidities with excessive doses (209). That calcium absorbed from the intestine can temporarily raise the serum calcium there is now no doubt (273, 427, 228, 24), although intravenous administration is certainly more effective (258, 304). There is little evidence (416) that parathyroid extracts, given by injection, can improve absorption of calcium from the intestine.

The curative action of irradiated ergosterol upon rickets can be demonstrated in parathyroidectomized rats (392). But a more urgent question is, how far can irradiated ergosterol, or less potent preparations of vitamin D, prevent tetany after parathyroidectomy? The earlier students of this subject, which has recently engaged a great deal of attention, were cautious in drawing conclusions, and inclined chiefly to emphasize the finding that treatment with the vitamin *before* operation mitigated the symptoms of tetany afterwards (265, 522, 266). Vitamin therapy was found successful in post-operative tetany (71, 479, 254) and infantile tetany (167, 217), but not in idiopathic tetany (317) in human subjects. But all these data are not decisive on the main point at issue, which is, can vitamin D replace the parathyroid hormone, or does it act by stimulating parathyroid tissue? Failures to relieve the tetany of parathyroidectomy have been reported many times (186, 518, 216); on the other hand, marked success has also been reported (67, 69, 131, 219, 414). Jones, indeed, reports (266) that with large doses of irradiated ergosterol he was able to raise the serum calcium of a dog in tetany, until it reached the astonishing figure of 47.3 mgm. per cent, with inorganic phosphate 19 mgm. per cent; a transient hyperphosphatemia has been observed by others also. Taylor has insisted on the great care that is necessary to remove all the parathyroid tissue in order to obtain a crucial experiment. In a recent paper (498) he reports that after ordinary thyroparathyroidectomy in dogs, large doses of irradiated ergosterol (1 cc. of 10,000 D) have a dramatic effect in relieving severe tetany, acting within an hour, and that two such doses may indefinitely prevent the reappearance of tetany. But in animals in which a free dissection of all the tissues of the neck had been carried out, from the hyoid to the sternum, the tetany was extremely refractory to this treatment, and it appears that the tetany was usually fatal; some of the animals, however, survived, and it was possible to produce hypercalcemia in them by further dosage with irradiated ergosterol; it is not clear whether this is attributed to the failure of even the drastic operation described to remove all the parathyroid tissue. Spiess, Wilson and Stringham (476) also performed very complete dissections, but found that viosterol raised serum calcium and urine calcium and decreased galvanic excitability nevertheless. More information as to the distribution of accessory parathyroid tissue in the dog is certainly required.

Irradiation with ultraviolet light has also been found to reduce tetany in thyroparathyroidectomized rats (270) and dogs (490); in normal

rabbits it induces hyperplasia of the parathyroids (172), which, however, is not accompanied by changes in serum calcium (165). On the other hand, on a diet deficient in vitamin D, the parathyroids of chicks do not develop normally without exposure to both visible and ultraviolet light (226).

In 1928 Pfannenstiel (401) and Kreitmair and Moll (292) found that very large doses of irradiated ergosterol had a toxic effect on normal animals, and this discovery awakened very widespread interest and led to intensive work and a flood of publications on the subject. Susceptibility to hypervitaminosis varies from species to species, and is in general parallel to susceptibility to the parathyroid hormone (498). Chickens, however, may suffer severely in hypervitaminosis, but are almost unaffected by large continued doses of parathyroid extract (278). There is hypercalcemia and hyperphosphatemia (293, 207, 530). There is, as usual, a difference of opinion as to which fraction of the calcium is most affected (161, 317). The excretion of calcium in the feces is reduced, no doubt because absorption is improved (530), but the increase in urinary calcium more than compensates for this, so that a negative calcium balance is the rule (73, 529); phosphorus excretion is also increased (530, 526). It is usually supposed that some part of the excess calcium comes from the bones, since although it is harder to obtain hypercalcemia and toxic symptoms on a low calcium diet (530, 267, 46, 209) it is not impossible, and hypercalcemia may even be obtained in eviscerated animals (497). The evidence that calcium is actually taken from the bones (217, 238, 465) has been criticized by Jones and Robson (268), who point out that as the experiments have been carried out on growing animals, with controls of the same age, they prove failure of calcification to continue rather than solution of deposited calcium; the latter effect they could not demonstrate in adult rats. So far, then, the effects of irradiated ergosterol in excess are similar to those of excessive parathyroid hormone, or at least there is no serious divergence. But it has been pointed out that the rise in blood phosphate is observed much earlier, and at lower calcium levels, than is the case in parathyroid overdosage. Further, there is, as well as possible rarefaction of bone, a hypercalcification of certain areas (232, 209) which is not clearly seen in parathyroid overdosage and recalls osteitis deformans rather than osteitis fibrosa (hyperparathyroidism). The metastatic calcification which is so characteristic of the hypervitaminosis on high calcium intake may be imitated by supply of very large amounts of calcium, or with parathyroid extracts (249, 305), but it has

been said that the sequence of events in hypervitaminosis is not simple, and that irritative and toxic effects precede calcification (465). Taylor (498) believes that the two pictures are entirely similar, that in dogs overdosage with irradiated ergosterol leads to death in the same way, with the same changes in blood chemistry, and with the same post-mortem findings as does overdosage with parathyroid extracts; but he concedes that the first effect of the former, which is a transient *fall* in serum calcium, is specific. Similar transient falls have been reported after parathyroid administration (488, 445, 37) but this claim does not seem to be well founded (215). Taylor's conclusion is that in all probability there is "a direct stimulation of parathyroid tissue by the vitamin" when the dosage is sufficiently great, and suggests that this may be "a compensatory mechanism to withdraw calcium from the bones and increase the excretion of calcium when an abnormal amount of the vitamin enters the body." It may be added that it is widely thought that the toxic effects and the metastatic calcification are to be ascribed, not to the vitamin itself, but to some by-product of the irradiation of ergosterol, and that a partial separation may be obtained (246, 238), but the evidence for this view is not wholly convincing. In recent experiments in our laboratory (10), dogs received repeated intravenous injections of large amounts of irradiated ergosterol, and very remarkable variations were seen. In some cases there was a rapid rise in serum calcium, followed by a rise in inorganic phosphate, and death with pathological findings exactly similar to those of parathyroid overdosage, as Taylor (498) has said. In other cases, however, the serum calcium rose only after great delay; its rise was not followed by any marked increase in inorganic phosphate, and hypercalcemia was tolerated well, loss of appetite being the only significant symptom. In one dog the serum calcium was maintained above 20 mgm per cent, on one occasion for five days, on another for twelve. A hypercalcemia of this degree, induced by parathyroid, we believe to be invariably fatal. We incline to think that when the onset of hypercalcemia is sufficiently slow, the body has time to adapt itself to the new conditions, and the rise in inorganic phosphate, to which, as has been said, we attribute much significance in the production of the characteristic symptoms, does not take place. So also in clinical hyperparathyroidism, hypercalcemia is well tolerated and the phosphate is not elevated. Against this view we have the extraordinary results of Jones (266) already quoted, where enormous hypercalcemia and hyperphosphatemia was well tolerated; and our own finding (10) that the excretion of calcium increases sud-

denly, in comparison with the slow rise of serum calcium in such refractory dogs, at a serum calcium level of about 15 mgm. per cent, which does not support the idea implied above, that the kidney may become adapted to the task of excreting abnormal quantities of calcium. Taylor and his collaborators (498), moreover, maintain that diarrhea and other "symptoms of the gravest nature" may appear while the inorganic phosphate of the blood is not above normal levels.

The action of irradiated ergosterol *in vivo* upon the phosphatase activity of the bones, the kidney, and the plasma appears to be in all cases a reduction—in the rabbit (30, 388), fowl (197), rat and dog (498). Heymann (224) alone was unable to detect any effect; he believes, it may be mentioned, that separate enzymes are required for the hydrolysis of glycerophosphate and of hexosephosphate.

Bischoff (49) affirms that when parathyroid extract is injected in a dog during hypercalcemia induced by irradiated ergosterol, it displays a paradoxical action and lowers the serum calcium. This does not seem to fall into line with other findings on combined actions (232, 367), and a repetition of Bischoff's work led (10) to the contrary result, that the response of the serum calcium to parathyroid was abnormally great.

The relation of other endocrine glands to calcium metabolism. In the numerous attempts that have been made to find an endocrine function for the thymus, much attention has always been made to its possible connection with calcium metabolism. Thus Uhlenhuth (516) found that feeding thymus to salamander larvae produced a tetany which was relieved by calcium (but this hardly proves that the tetany was due to calcium deficiency in the first instance). Riddle (419) found a group of pigeons which, apparently as an idiopathic disorder, laid eggs deficient as to shell and albumin; and he discovered that by feeding thymus, and in this way only, the condition was corrected. It has been stated that a similar condition may be produced experimentally by thymectomy, of which osteomalacia is also said to be a consequence. Riddle (421) has criticized these claims, and believes that thymectomy does never, and can never, give valuable information, because thymic function is shared by tissues in many parts of the body. This objection presumably does not hold against experimental atrophy of the thymus produced by thymotoxic serum, a procedure said to be followed by decalcification. Harris (208) has briefly summarized the evidence linking the thymus with calcium metabolism, and includes the claims that implants of thymus increase the density of bones, and that there is atrophy of the thymus and reduction in the number of lymphocytes in

hypervitaminosis D. Thymectomy has no influence on the production or cure of rickets in rats (392). Nitschke (376) and Reiss (417) have prepared acid extracts of thymus which lower serum calcium in the rabbit, but not, apparently, in the dog (we have already expressed our distrust of the rabbit as an experimental animal in work of this type), and which may cause hypercalcification in the mouse. We remain unconvinced that any definite rôle of the thymus in calcium metabolism has been established, though it must be admitted that the evidence, such as it is, all points in one direction.

That the thyroid gland has some connection with calcium metabolism appears to us to be absolutely certain. Parhon (394) noted an increased excretion of calcium in rabbits treated with thyroid; rarefaction of bones has been noted several times in cases of exophthalmic goitre of long standing (43, 405, 16), and calcium excretion is said to be high (294). But the work of Aub, Bauer, Heath and Ropes (16) put the matter on a much firmer footing. They showed that the excretion of calcium was on the average 250 per cent above normal in cases of hyperthyroidism, and that it could be increased in normal individuals by administration of desiccated thyroid substance; nor was this effect a secondary result of high basal metabolic rate, since in subacute bacterial infections it could not be seen. The picture differs from that of hyperparathyroidism, since there is a marked increase in calcium excretion by the intestine, as well as by the kidney. The excretion of phosphorus was also increased so that solution of bone could be assumed to be taking place. In myxedema, the calcium excretion was abnormally low, and was increased by thyroid treatment. It has not been found, in general, that the serum calcium departs from the normal level in thyroid disease; but thyroid treatment improves the general condition and raises serum calcium in parathyroid tetany in human subjects (16) and in dogs (296, 297). The faulty calcification seen in parathyroidectomized rats is less severe if the thyroids are also removed (202). The plasma phosphatase is somewhat above normal in hyperthyroidism (277). Aub (16) believes that the thyroid hormone is the most potent stimulus to calcium excretion known (though Deuel and his collaborators (132) did not note any change after a moderate dose of thyroxin) and considers that it has "a direct stimulating catabolic effect on the calcium deposits in the bones." Although the excreted calcium helps to neutralize the acids simultaneously produced, it is not probable that the mobilization of calcium is merely a defensive mechanism against general acidosis; though it is conceivable that *local* acidosis may serve to release

calcium from the bones (7). We may point out that in his experiments, and indeed in all such experiments except those of Kunde (297), pure thyroxin has never been used *by itself*, and that it remains possible, however improbable, that some other constituent of the gland is responsible for the effect. It has been observed that hyperthyroidism increases sensitiveness to the parathyroid hormone (37, 121). Cameron and Carmichael (81) observed tetany in rats fed with thyroid, and attributed this at first to hyperpnea, but later (84) to a diminution of the blood flow and general activity in the thyroid and *parathyroid* glands under the influence of the exogenous supply of thyroid. Could it be supposed that increased calcium excretion led at last to a fall in serum calcium to tetanic levels? It has been said that serum calcium may be low in cases of toxic goitre (522), and in experimental hyperthyroidism in dogs (393). But Kunde (295) noted occasionally low serum calcium in cretin rabbits, and a rise when thyroxin was given. In hyperthyroid dogs, however, she (297) noted a rise in acid-soluble phosphorus with no change in calcium. Recently Baumann (29) has shown that in the experimental goiter with hypothyroidism produced by feeding cabbage to rabbits there is marked retention of calcium, magnesium, and phosphorus, while a normal balance is restored when involution of the gland is produced by iodine. This effect is no doubt different from the rise in serum calcium reported to follow the feeding of cabbage to rabbits (122, 123), which cannot always be obtained (274) and is at best but a transient phenomenon.

Acid extracts of spleen are reported to behave like those prepared from the thymus (377, 417) in lowering serum-calcium in the rabbit. It has been said that splenectomy lowers, and feeding desiccated spleen raises, the serum calcium in the dog (70). Beside these contradictory data we may place a mention of the high serum calcium in cases of polycythemia vera (72).

The theory put forward by S. G. Zondek that the effects of sympathetic stimulation are due to a setting free of calcium ions from the nerve-endings, and that potassium plays a similar rôle for the parasympathetic, has attracted a good deal of attention in Germany, though its experimental basis seems to us of the flimsiest. He (541) believes that a rise in serum calcium can be demonstrated after sympathetic stimulation. But it is reported that adrenalin causes a slight fall in serum calcium (45, 308, 300) which is surprising, since it undoubtedly lowers inorganic phosphate by stimulating ester synthesis, and tends to produce acidosis; Vollmer (521) believes that there is an initial rise in calcium. The

weakness of the experimental justification for the use of adrenalin in osteomalacia has been pointed out by Hunter (256). Sympathectomy is not found to affect the serum calcium level (300, 17) nor the response to parathyroid hormone (17). Section of the splanchnic is said to cause a marked fall in serum calcium for a week or more, while section of the vagi occasionally has the contrary effect (39). Adrenalectomy in dogs may lead to hypercalcemia, (91, 429) but this may depend on absence of the cortical hormone rather than of adrenalin. Extracts have been obtained from the cortex which lower serum calcium in the rabbit (361).

Since the administration of insulin, or of glucose, or both, leads to a fall in inorganic phosphate ascribable to ester synthesis, it is to be expected that a rise in serum calcium may sometimes be observed. So the demonstration of this effect in rabbits (128) does not impugn the specificity of the parathyroid hormone; as a matter of fact, Culhane (122) was unable to confirm these experiments, and ascribed the effect to the use of cabbage in the diet. Both insulin (413) and glucose (412) have an effect in temporarily alleviating parathyroid tetany in dogs, and in normal dogs (66) and human subjects (144, 155) a rise in serum calcium has been observed on several occasions.

It has been said that in obstructive jaundice in man the serum calcium may be slightly below normal (474, 76); Cantarow found the response to parathyroid unaffected, but calcium excretion increased (86). In experimental jaundice in dogs serum calcium is unchanged (474, 512) or lowered (146, 76); the claim that it is elevated (279, 280) seems to be based on faulty experimental technique, yet jaundiced dogs succumb less readily to parathyroid tetany (68). Emerson (146) found calcium raised in dogs with biliary fistula (cholecystonephrostomy) and believes that the diffusible fraction is chiefly concerned. Injections of bile or bile salts have given contradictory results (11, 126).

The relation of the ovary to calcium metabolism is more important both theoretically and practically. It has been said that at the menstrual period there is a fall in calcium in the serum (342, 64), but others have seen no regular change (97) or a decrease before the period (383). That oestrus and pregnancy aggravate tetanic symptoms in parathyroidectomized dogs has long been recognized (326, 136). The serum calcium usually falls during pregnancy (345, 352, 536, 475, 212, 89, 3, 26) and electrical excitability is increased (449), even manifest tetany being not uncommon. This makes the view of Bokelmann and Bock (55), that the diffusible calcium is increased, unlikely; and it has not been confirmed directly (212), though a rise in cerebrospinal fluid calcium

is reported (89). It is thought that these effects may be noted too early for the influence of the demands of the fetus for calcium to be significant. Aub and his colleagues (26) found that the excretion of calcium was not reduced to compensate for these demands. Negative calcium balance may be observed in pregnancy (331), but not always (115, 170); lactation, on the other hand, probably always entails a loss of calcium from the body (170, 252) so that serum calcium may be lowered again (350, 3). Fehling introduced oöphorectomy as a treatment for osteomalacia, which has been much discussed, some believing that the benefit is due merely to avoidance of pregnancy with its drain on the maternal calcium reserves, others that a specific effect is involved. Hoyle (245) is inclined to think that the benefit may sometimes be seen so rapidly that the former view is excluded; but Maxwell (344) in his studies of osteomalacia in the East, where it is so commonly observed, regards the rôle of the ovary as unimportant. Mirvish found that an ether-soluble fraction from ovarian extracts lowered serum calcium in rabbits (359) and in man (360), irrespective of sex; and Reiss (415) obtained similar results on rabbits. These extracts doubtless contained oestrin, but were very far from purity. Extracts of suprarenal cortex (361) and testis (362) also lower the serum calcium in rabbits, and, as has already been mentioned, so also do extracts of thymus and spleen; there is therefore no justification for supposing these effects to be due to specific hormones such as oestrin (theelin). In the bird the whole question is complicated by the demand for calcium for the formation of the egg-shell. Riddle (420) found that the blood calcium of the pigeon rose very high, up to 20 mgm. per cent, before and during ovulation. Recently Sun and McOwan (486) have noticed in hens that the blood calcium rises to a peak, even to 25.6 mgm. per cent, at the time when the egg weighs 10 to 20 grams, but before it has attained its full size. They also noted histological changes in the parathyroids at this period.

The preparation and assay of potent parathyroid extracts. Acid extracts of parathyroid glands were prepared by Berman (41) and Hanson (204) which, in the latter case at least, were not devoid of activity. Conclusive proof of the potency of such extracts was furnished by one of us (103), and purification was carried further. The method as used in our laboratory (106) consists in extracting the fresh or frozen glands with 5 per cent hydrochloric acid for 30 to 60 minutes at 100°C. The extract is treated with excess of alkali to bring it to pH 8 or pH 9, and is then neutralized at pH 5.5 and filtered. The precipitate is redis-

solved and again filtered. The combined filtrates are saturated with sodium chloride, the precipitate dissolved in NaOH, and the active fraction precipitated at pH 4.8. This isoelectric precipitation is repeated several times. The product when dried contains 15.5 per cent nitrogen, and traces of iron and sulphur. The weight of one unit is approximately 0.3 mgm. The substance is amorphous, soluble in water and in 80 per cent alcohol, but insoluble in ether, acetone and pyridine; it gives all the common protein colour reactions, and is precipitated by picric and picrolonic acids. It is destroyed by heating for an hour with 10 per cent HCl or 5 per cent NaOH, and by incubation with pepsin and trypsin; it does not dialyse through collodion.

Quite similar extracts were prepared by Hjort (227) and Fisher and Larson (159). Davies, Dickens and Dodds (128) claimed that a preparation made according to the Dudley technique for insulin (using picric acid and acetone) raised serum calcium in rabbits, but such preparations were found inert in dogs (159). Tweedy (514) using weaker acid and a shorter period of extraction, removed some impurities from the extract by adding one volume of acetone; he precipitated the active principle with trichloracetic acid, extracted the precipitate with chloroform, and dissolved it in saline. Recently (515) he has made a 20 per cent solution of his preparation in 90 per cent aqueous phenol solution, precipitated impurities by adding alcohol to a concentration of 75 per cent, and finally precipitated the active principle by adding ether. His preparation, unlike ours (106), still gives a positive Molisch reaction, as well as other protein colour-tests. It does not appear that it is more potent than our preparation. A recent study by Doctor Allardyce in our laboratory (9) has shown that while *both* acidity and elevated temperature are required in the preparation of active extracts, the treatment hitherto used has been too drastic. The best results were obtained with 1.5 per cent HCl for 45 minutes; with stronger acid the maximum activity was obtained in a shorter time, but destruction outruns extraction and less potent preparations are obtained. Attempts made to apply the methods so successful in the case of insulin, both the brucine-pyridine method of Abel and the saponin method of Harington, failed; in the latter case, indeed, the activity was destroyed. Attempts to utilize extraction with absolute alcohol, or aqueous butyl alcohol, also failed.

The unit of potency of such extracts was defined (105) as one one-hundredth of the amount of extract which will produce an average increase of 5 mgm. per cent in the blood serum calcium of normal dogs

of approximately 20 kilograms weight over a period of 15 hours, using subcutaneous or intramuscular injection. It is clear that in the absence of any standard preparation for comparison, and with only the limited amount of information as to interfering factors which is cited below, the accuracy of this method of assay can never be very great. It is necessary to use a number of dogs for each test—the more the better. This depends not only on variation between individuals, but also on irregular variations in the response of any individual tested on different occasions (9). Burn (79) comments that "no information is available concerning the procedure which is followed when the average rise is less than 5 mg., being equal perhaps to 2 mg. It is clearly of importance to know whether the dose is then considered to contain only 40 units." But in fact charts 3 and 4 of the original paper (105) do answer this question, showing that the response is proportional to the dose in this range. We have calculated that the ratio of dose to response, for the fifteen experiments included in the second of these charts, shows a 22 per cent standard deviation from the mean. This signifies that once in 22 trials the expectation that response will be proportional to dose will be in error by 45 per cent or more. It is obvious, however, that the error introduced by inaccuracy in serum calcium determination will be greater if the rises measured are small; on the other hand, the rise is no longer proportional to the dose when the dose is very large, rises greater than 8 mgm. per cent being seldom observed. A regular relation between the effectiveness of a given dose and the weight of the experimental animal has not been observed, and the remark that "10 units . . . . is the amount necessary to produce in fifteen hours a rise of 5 mg. per 100 c.c. in a 2 kg. dog" (481) is based on an assumption for which there is no experimental justification. Hanson (205) has proposed as a unit one one-hundredth of the amount required to produce a 1 mgm. rise in the serum calcium of 15 kilo dogs 24 hours after parathyroidectomy. It has frequently been felt that parathyroidectomy increases sensitiveness to the hormone, in the dog (104, 227, 330) and in the rat (513), but a careful examination of the point (105) did not support this view. Burn (79) suggests that the rise of serum calcium in two hours after intravenous injection into cats, noted in the experiments of Stewart and Percival (481), should be used for assay. In our hands, as previously noted, such experiments have given no appreciable rise in serum calcium (9), and the method is clearly not of universal applicability. No technique for assay can be regarded as satisfactory unless

the possibility of changes in plasma pH, or in blood sugar concentration, is taken into consideration; hence anesthesia, or struggling, or asphyxia, must as far as possible be excluded.

The effect of diet upon assay deserves consideration—more, indeed, than it has yet received. Macleod and Taylor (330) found that a dog on a meat diet was more susceptible than a dog on a diet of bread and milk; but on repeating the experiment the reverse result was obtained. In our laboratory, it was found (9) that on a diet consisting chiefly of porridge (rolled oats) the responses were small and irregular, and not improved by adding calcium or cod-liver oil; whereas on a diet of beef heart good results were obtained. It has been noted that oatmeal converts a just-sufficient diet into a rachitogenic diet (351, 235, 175) and Mirvish (363) has claimed that a hydrochloric acid extract of oatmeal lowers serum calcium in the rabbit. Such extracts, however, were found to be inert in dogs and not to influence the response to parathyroid extract (9).

When these extracts were first described (102, 103), the name "parathyrin" was given to the active principle. This was subsequently withdrawn, on the ground that it had already been used for a commercial preparation. But today we find that the trade name of another commercial preparation, made by the method described (106), is creeping into use as a synonym for the parathyroid hormone. One or two writers have coined the illogical term "parathyroxin." We suggest that the matter may be allowed to rest as it is until some really striking advance in the purification of the active principle has been made.

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PHOSPHATASE IN GROWTH AND DISEASE OF BONE

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During the last two decades, many domains of enzymic study have been well surveyed, cleared and developed, and in not a few "systematic mining" is now in progress. But it is inevitable (to extend Gowland Hopkins' (1917) well-known metaphor) that other fields, perhaps more difficult of access but in which nuggets are still to be found on the surface, will attract more and more the pioneer. Whereas the attention of many workers in every country and in all branches of physiological science has been occupied hitherto with the discovery and identification of new enzymes, their purification and probable chemical nature, their specificity, dependence on activators and inhibitors and their general physicochemical behaviour, it is likely that in the near future more attention will be paid to fields at present hardly entered, among which may perhaps be specified those of the actual *in vivo* function and significance of intra-cellular enzymes, the methods of and conditions controlling the synthesis of these agents from inactive material by the organism, their evolutionary import, the meaning of their sometimes apparently fortuitous distribution, and the development of a new type of therapy or even of constructive control of the organism by regulating the rate or degree of specific enzymic activity within the living cell.

Although all biologists interested in the inner economy of the cell will agree that intracellular enzymes must play a definite and specific part in the everyday processes of tissue breakdown and repair, or of storage and secretion, the reversibility of which processes is one of the essential concomitants of life, there are very few cases on record, if indeed there are any, in which the biological function of an intracellular enzyme has been defined with some degree of certainty. This point may be illustrated by a short consideration of liver amylase, an enzyme which has been frequently investigated, and which must undoubtedly, *in vivo*, play a catalytic rôle in the breakdown of glycogen to glucose, and, somewhat less certainly, in the synthesis of liver glycogen from glucose, lactic acid and other compounds. *In vitro* the process of breakdown

has been well explored and much clear-cut information is to be found in the literature. But as yet no enzyme has been cleanly separated from liver tissue which will either hydrolyse glycogen to glucose or perform any synthesis of glycogen from any source (see also Raper 1930).

In the case of phosphatase, particularly animal, or more strictly, *mammalian* phosphatase, we are, perhaps, in a more fortunate position. We know something of its distribution, its substrates, the effect of various external conditions on its activity, and the influence upon it of certain activators and inhibitors. We also know something about its synthetic activities and, as will be seen from the following pages, there is now a considerable body of evidence pointing to its specific biological rôle in the deposition and maintenance of the mineral salts present in the bones and teeth.

THE DISCOVERY OF THE ENZYME. Enzymes hydrolysing phosphoric esters have been known for some years. Suzuki, Yoshimura and Takaishi in 1907 reported the existence in rice and wheat bran of an enzyme capable of decomposing phytin (the calcium-magnesium salt of inositol hexaphosphoric ester) with liberation of inorganic phosphate. McCollum and Hart (1908) demonstrated that animal tissues also (calf liver and blood, but not, apparently, muscle or kidney) would hydrolyse phytin in the same way. Plasmata derived from various organs of the dog were found by Levene and Medigreceanu (1911) to hydrolyse nucleotides with the production of free phosphate, intestinal mucosa and kidney tissue yielding particularly active extracts. Grosser and Husler (1912) found that an enzyme which would hydrolyse glycerophosphate was widely distributed in animal organs; the order of activity being kidney and intestine (highest), lung, liver, spleen. Blood and cardiac and skeletal muscle were inactive. Both Merck's synthetic glycerophosphate and that obtained by hydrolysis of lecithin were attacked almost equally well. Euler (1912) showed that mammalian intestinal mucosa and kidney would hydrolyse Harden and Young's hexose diphosphate, and that if this substance were fed to a dog it was almost completely hydrolysed. It was found by Harding (1912) that ricinus lipase and emulsin would hydrolyse the same substrate, but that pancreas was almost inactive.

Plimmer (1913) confirmed these findings as regards the hydrolysis of both glycerophosphate and hexose diphosphate by extracts of intestinal mucosa, kidney and lung, and extended the work to other phosphoric esters and enzyme preparations. Tomita (1922) showed that tissues from both warm- and cold-blooded animals (carp) would hydrolyse

Neuberg's hexose monophosphate, the muscle being least active of the tissues investigated. (For further references, particularly to the occurrence of phosphatases in plant tissues, which is not considered in this review, Oppenheimer (1925 and 1929) should be consulted.)

Robison (1923) observed that the readily soluble barium and calcium salts of the hexose monophosphoric ester first obtained by Harden and Robison (1914) were readily hydrolysed by emulsin with the formation of a precipitate of barium or calcium phosphate. This suggested to him that some such reaction might conceivably be concerned in the deposition of calcium phosphate in the ossifying zone of cartilage during bone formation. Hexose-phosphatase had not, up to this time, been reported as occurring in bone. Robison was able to show that this enzyme was present in the ossifying cartilage of young rats and rabbits, non-ossifying cartilage possessing less than one-tenth of the hydrolytic power of ossifying cartilage. Extracts of ossifying cartilage (femur epiphysis of young rabbit) would hydrolyse glycerophosphates in much the same way. The kidney contained an active enzyme also, but the activity of kidney was rather less than that of an equal weight of epiphysial tissue. The enzyme in bone was not confined, however, to the epiphysis but was present in considerable, though lesser, quantities in the shafts of long bones as well. The surprising and striking fact that such a relatively inert tissue as bone should contain this active enzyme, so conveniently situated for the deposition of calcium phosphate, pointed directly to the probability that the enzyme played some specific part in bone formation. Up to the date of the discovery of this enzyme in bone, the cause of the deposition of calcium phosphate in this tissue and its non-deposition elsewhere had not been satisfactorily explained. The hydrolysis of some phosphoric ester, the calcium salt of which is readily soluble in water or plasma, by an enzyme present to a greater extent in ossifying cartilage than in any other tissue, leading to the *local* production in the calcifying zone of an inorganic phosphate ion concentration considerably higher than that in the blood, offered an explanation of calcification which seemed to fit the facts much more closely than the theory of specific adsorption by ossifying cartilage (Pfaundler, 1904; Freudenberg and György, 1920–1923) which was one of the less unsatisfactory hypotheses in the field in 1923. (For discussions of some of these hypotheses see Watt, 1925, 1927, 1928, and Leriche and Policard, 1928.) These observations of Robison form the starting point for a series of researches which have thrown increasing light on the mechanism of calcification in ossifying tissues.

Before dealing further with the biological significance of phosphatase, it is advisable to consider some properties of the enzyme.

PROPERTIES OF MAMMALIAN PHOSPHATASE. a. *Distribution.* The determination of the quantitative distribution of any enzyme in living tissue is, for several reasons, a problem of a quite different order from that of the distribution of any particular element or compound. In the case of an enzyme, apart from the fact that evidence of its presence and activity is at best only second-hand, there are other difficulties. Thus 1, it may be found impossible to remove the enzyme from the main bulk of the tissue by any available method of extraction, so that large quantities of associated tissue constituents of unknown influence on the enzyme's activity accompany it; 2, extracts of different tissues, even if prepared similarly will probably contain, in addition to the enzyme, greater or lesser amounts of accelerating or inhibiting substances; 3, no tissue with the exception of blood plasma is even approximately homogenous, so that the activity of the enzyme will undoubtedly vary from one part of an organ or tissue to another, and from one group of cells in that organ or tissue to another; thus an organ extract may furnish an average value for enzymic activity which may give no idea whatever of the widely differing activities in the organ from one group of cells to another—a clear example of this in the field under review is the finding that kidney cortex contains much more phosphatase than does the medullary tissue or the pyramids (Kay, 1926); 4, the activity of the enzyme will vary with the pH of the extract, which pH may not be the same for extracts of different tissues, or of homologous tissues which have been allowed to autolyse for different lengths of time; 5, the functional pH for most intracellular enzymes is unknown, so that it is not possible to state what the relative activity of the same enzyme in different tissues *in vivo* will be, even if we can determine the relative activity *in vitro;* 6, the choice of substrate for the enzyme may be difficult if the "natural" substrate is unknown.

It is impossible to avoid all these potential sources of error; we can perform the determinations neither under strictly natural nor strictly artificial conditions, and a compromise is inevitable.

The distribution of phosphatase in animal tissues has been examined by several workers, with results which, in view of the difficulties just outlined, do not disagree seriously. The relative *glycerophosphatase* activity of various tissues is shown, as far as is possible from the available data, in table 1.

From the figures given in table 1 the general conclusion may be drawn

that, taking the tissues of adult animals, the glycerophosphatase activity of the intestinal mucosa is highest per unit weight of wet tissue, then follows the kidney (average for the whole organ, but the cortex taken separately would probably still be much below intestinal mucosa). The

TABLE 1

Relative glycerophosphatase activity of tissue extracts prepared under similar conditions from various mammalian tissues

	REFERENCE						
	Grosser and Husler (1912)	Forrai (1923)	Robison (1923)	Kay (1928, 1)			Kay (1931, 2)
	Cat	Man	Young rabbit	Adult			Adult rat—average of 4
				Rabbit	Cat	Man	
Tissue:							
Intestine	100	100					
Duodenum				50*	93*	57*	46
Jejunum				100*	100*	85*	33
Ileum				53*	81*	100*	15
Colon				17*	34*	27*	6
Kidney	100	58	36	33	38	35	100
Ossifying cartilage			100				
Whole bone				20	10		76
Liver	16	16	43	12	4	6	4
Pancreas		8	11				
Spleen	Trace		25	18	3	7	18
Lung	62			10	26	7	20
Blood	0		14				1
Thyroid		19					21
Thymus		8					
Suprarenal		24				10	19
Testis		12					13
Brain cerebrum				3	3	4	7
Cardiac muscle	0			1	1		5
Skeletal muscle	0		4	1	1		2
Artery					Nil	Nil	
Stomach				1*	5*	4*	5

* Mucosa only.

Most active tissue rated in each animal as 100, activity of other tissues computed on this scale. Figures only approximate, and do not indicate relative activity from one species to another, but only from one organ to another in the same species.

Phosphatase is also present in small quantities in milk, bile, urine and blood plasma (Demuth, 1925).

activity of whole adult bone per gram is less than that of the kidney, but it must be remembered that in whole adult bone, not only is some 35 to 60 per cent[1] of mineral salts present, but the fraction of the organic portion actively concerned with maintenance of the bone structure must be small. If it were possible to separate completely the cells concerned in bone maintenance from the rest of the structure, there is reason to believe that the phosphatase content of this group of cells would be as high, if not higher than that of any other tissue in the adult body.

In the young and particularly in the fetal mammal, the relative quantities of phosphatase in the various organs and tissues show a very

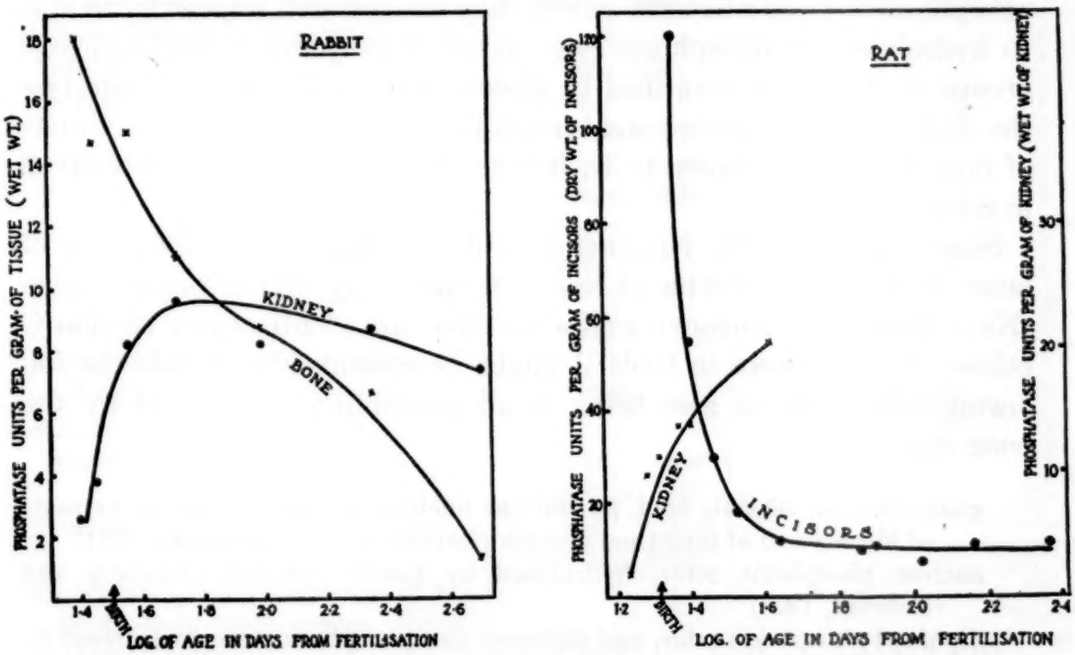


Fig. 1. Change in the phosphatase activity of rabbit tissues and rat tissues with increasing age. (From Kay, 1926, and recent experiments in this laboratory by Dr. A. S. E. Mackenzie.)

different picture. Here the amount of phosphatase is higher in the rapidly growing whole bone and tooth than in any other structure (see fig. 1). The growing bone diminishes in phosphatase content after birth of the animal (rabbit) whilst the kidney phosphatase increases as the organ becomes fully functional.

Although it is intended to limit this discussion to the properties of the phosphatases of mammalian tissue, a recent paper (Bodansky, Bakwin and Bakwin, 1931) dealing with phosphatase in the tissues of teleosts and elasmobranchs has con-

[1] Calculated on the fresh tissue.

siderable interest. It is found that teleosts, on the whole, have considerably more phosphatase in the kidneys, somewhat more in the skeleton, and a little more in the intestinal tract than elasmobranchs. A definite though small phosphatase activity occurs even in the non-ossifying skeleton of the elasmobranchs, but the activity per gram in the kidney and intestine in both cartilaginous and bony fish is very much greater than in the skeleton. The method of phosphatase determination used does not, unfortunately, permit of close comparison of these results with the findings for mammalian tissues, but it would appear that the activity of the fish tissue is markedly less than that of corresponding mammalian tissue.

b. *Enzymic Characteristics of Mammalian Phosphatases* (with particular reference to the bone enzyme). 1. *Substrates.* The mammalian phosphatases, from whatever source they are derived, appear to be able to hydrolyse any phosphoric ester in which only one of the hydroxyl groups of the acid is esterified by almost any alcohol, but to hydrolyse the di-esters of phosphoric acid much more slowly, if at all. Extracts of bone have been shown to hydrolyse all the phosphoric esters given in table 2.

Since there is fairly convincing evidence that the phosphatases of bone, kidney and intestine at least, and probably that of blood plasma (Kay, 1930a) and leucocytes (Roche, 1931) are identical (see section C below) the list shown in table 2 might be extended to include the following substances as also being in all probability hydrolysed by the bone enzyme:

guanylic acid, inosinic acid, pyrimidine nucleotides, hydrolysed by extracts of kidney and of intestinal mucosa (Levene and Medigreceanu, 1911)

sucrose phosphoric ester, hydrolysed by kidney extracts (Neuberg and Behrens, 1926)

diphenyl phosphoric ester, and diphenyl pyrophosphoric ester hydrolysed by kidney extracts (Neuberg and Wagner, 1926)

methyl, methylpropylcarbinyl, cetyl, cholesteryl phosphoric esters, di-o-cresyl di-m-cresyl pyrophosphoric esters hydrolysed by kidney extracts (Neuberg and Jacobson, 1928)

acetonyl, propyl, isopropyl, phenyl, benzyl, cresyl, diethyl, diglyceryl and dicresyl phosphoric esters hydrolysed by kidney extracts (Akasawa, 1929)

The bone enzyme will also hydrolyse some 30 to 40 per cent of the organic acid-soluble phosphorus compounds of whole blood (Kay and Robison, 1924a) and apparently all the organic acid-soluble phosphorus compounds in blood plasma (Martland and Robison, 1926). It will also hydrolyse a large fraction of the acid-soluble and acid-stable organic phosphorus compounds of muscle (Kay and Robison, 1924b), kidney (Kay, 1926), liver (Kay, 1931a) the chemical composition of which com-

pounds is still somewhat obscure—adenylic acid, and possibly other nucleotides, glycerophosphoric acid, and hexose and pentose phosphoric acids in varying proportion are likely to be present.

TABLE 2

Phosphoric esters known to be hydrolysed by bone phosphatase

SUBSTRATE	REFERENCE	REMARKS
Glycerophosphoric ester	Robison, 1923	Optimum pH 8.4–9.4
Hexose monophosphoric ester (mixture of glucose-6-phosphate and fructose-6-phosphate)	Robison, 1923	Optimum pH 8.4–9.4
Hexose diphosphoric ester (Harden and Young)	Robison, 1923	
Phosphopeptone from casein	Rimington and Kay, 1926	
Monoethyl phosphoric ester	Martland and Robison, 1927	
β-methyl-hexoside diphosphoric ester	Morgan, 1927	
Guanylic acid from yeast nucleic acid	Kay, 1928a	Optimum pH 9.2
Pyrophosphoric acid (phosphoryl phosphate)	Kay, 1928b	Optimum pH 7.6
α-methyl-hexoside diphosphoric ester	Morgan and Robison, 1928	
Trehalose monophosphoric ester	Robison and Morgan, 1928	
Monoglycol phosphoric ester (β-hydroxyethyl dihydrogen phosphate)	Kay, 1931a	Optimum pH 8.8–9.0
α- and β-glycerophosphoric esters	Kay, 1931a	Optimum pH varies with conditions, usually 8.9–9.4
Fructose monophosphoric ester (fructose-6-phosphate)	Roche, 1931	
Monophenyl phosphate	Roche, 1931	
Adenylic acid from yeast nucleic acid	Roche, 1931	
Diphosphoglyceric acid (Greenwald, 1925)	Roche, 1931	

During the last year an interesting exception to the general rule that orthophosphoric esters are hydrolysed by the animal phosphatases has been reported by Jacobson and Tapadinhas (1931) who have found that

no hydrolysis of menthyl or dimenthyl orthophosphoric ester is brought about by active samples of phosphatase from various sources, e.g., sheep and rabbit liver and kidney. That the effect is not one of simple steric hindrance due to the large terpene radicle is shown by the fact that bornyl orthophosphate is hydrolysed by the same enzyme preparations.

The hydrolytic activity of bone phosphatase, therefore, is *not* specific for any particular phosphoric ester or type of ester, e.g., a *hexose* phosphate. Almost every monophosphoric ester which has been presented to it is hydrolysed by the bone enzyme, and any such ester which forms a soluble calcium salt will serve as a substrate for calcium phosphate precipitation.

2. *Hydrogen ion concentration and phosphatase activity.* Most of the phosphoric esters which have been investigated as substrates for the phosphatases from bone (or ossifying cartilage) or from other mammalian tissues are hydrolysed at increasing rates as the reaction of the medium becomes more alkaline. An optimum rate is usually reached in the neighbourhood of pH 9 (see table 2). This optimum, however, depends on the conditions of hydrolysis as well as on the nature of the substrate, and, like the optimum temperature for enzymic hydrolysis, is probably dependent to a large degree on the rate of destruction of the enzyme. Phosphatase begins to be destroyed at pHs greater than 8.8, particularly so at body temperatures or above. For this reason the apparent optimum in the neighbourhood of pH 9 is almost certainly misleading, the true pH-activity optimum may be considerably higher. Values for the optimum as high as pH 9.9 have been reported by Akasawa (1929) for kidney phosphatase, and values of 9.6 and above have occasionally been observed both for intestinal and for kidney phosphatase in presence of high concentrations of substrate in the present writer's laboratory. It would appear likely that the rate of hydrolysis continues to increase with increasing alkalinity, and that the apparent optimum is set by the increasing rate of enzyme destruction. The enzyme is readily inactivated by acids at pHs of 5 or below.

Although it is doubtful whether pyrophosphates, uncombined with adenylic acid occur free in the tissues, pyrophosphate is the only substrate of known biological importance which is readily hydrolysed by the bone enzyme at a pH close to that of the tissues. The optimum pH for this substrate is in the neighbourhood of 7.6 (Kay, 1928b). Akasawa (1928) has reported optima in the same region for the action of kidney phosphatase on the "unphysiological" substrates diethyl, diglyceryl, diphenyl and dicresyl phosphoric esters.

The activity-pH curve for bone phosphatase, according to Martland and Robison (1927), has the form of the dissociation curve of a weak acid, p_K about 8.2, or the dissociation-residue curve of a weak base (compare Michaelis, 1911, 1920).

It is of interest that the same pH optimum of about 9 is obtained when mammalian phosphatase acts on the organic acid-soluble phosphorus compounds of kidney, blood or liver. The possible biological significance of this very alkaline optimum will be dealt with later.

Recent work of Roche (1931) has indicated a distinct difference with respect to hydrogen ion activation between the phosphatase of the red cells on the one hand and that of the plasma, the white cells, the kidney, intestine and bone on the other. The optimum pH for the activity of the red-cell enzyme is on the acid side of neutrality, namely, from pH 6.0 to 6.8, according to the substrate, and it acts more energetically on the α- than on the β-glycerophosphate, whereas the other mammalian phosphatases, with an optimum pH in the neighbourhood of 9, hydrolyse the β-ester more quickly.

For plant phosphatases the optimum pH is usually well on the acid side of the neutral point (Adler, 1915; Akasawa, 1928, 1929; Kobayashi, 1926; Němec, 1920, 1923; Fernandez and Pizarosso, 1928; Inouye, 1928; Kay and Lee, 1931). In unpublished experiments the present writer has found that the yeast- and taka-phosphatase optima (substrate β-glycerophosphate) are in a still more acid zone than those for soya bean or pea phosphatase.

3. *Reversibility of phosphatase action.* As the stock example of a balanced reaction, every student will cite the equilibrium between an ester and water on the one side and the acid and alcohol from which the ester is formed on the other.

$$\text{i.e., ester} + \text{water} \rightleftharpoons \text{alcohol} + \text{acid}$$

He will continue by saying that in very many cases the reaction in either direction proceeds so slowly that a small quantity of catalyst is required to be added to the system if any hydrolysis or synthesis is to be observed within a reasonable time. Such a catalyst may be a mineral acid, or an enzyme, and he will doubtless quote the classical work of Kastle and Loevenhart (1900) on lipase.

The reaction between glycerol and phosphoric acid belongs to this category of reversible reactions, but also requires the presence of a catalyst if either synthesis or hydrolysis is to be observed, at least at ordinary temperatures and pressures.

It had been known for some time that relatively low concentrations of inorganic phosphate had an inhibitory effect on the hydrolysis of phosphoric esters by phosphatases. Bodnár (1925) and Martland and Robison (1927) showed further that under certain conditions inorganic phosphate would disappear if tissue extracts derived from plant (Bodnár) or animal sources (Martland and Robison) were incubated with inorganic phosphate and an alcohol. On these grounds, synthesis of a phosphoric ester was postulated (though synthesis of pyrophosphate was not definitely excluded). The latter workers, using bone phosphatase, found that glycerol, glycol and mannitol certainly, and possibly glucose and fructose also, all in fairly high concentration, would cause this disappearance of inorganic phosphate. They did not, however, isolate any of the synthesised products. In 1926 the present writer had observed the same phenomenon with methyl alcohol, ethylene glycol and glycerol, using kidney or duodenal phosphatase as the catalyst, and was able eventually to isolate several grams of enzyme-synthesised esters, namely, the mono-phosphoric esters of glycerol and of glycol, in the form of the barium salts (Kay, 1928a; 1929a). The reaction between glycerol and secondary sodium phosphate proceeds so smoothly in presence of the duodenal enzyme that it makes a very suitable class experiment in enzyme synthesis for senior students (Kay, 1928d). The time relationships of synthesis and hydrolysis are shown in figure 2.

From figure 2 it is evident that whether inorganic phosphate or glycerophosphate is present at the start, approximately the same position of equilibrium is eventually reached provided that water and glycerol are present in amounts which are large relative to the total quantity of phosphorus. The position of equilibrium, and the speed at which it is reached are also seen to be dependent on the relative concentrations of glycerol and water.

In unpublished experiments in this laboratory it has been found that the optimum pH for synthesis with duodenal phosphatase, sodium phosphate and glycerol, at 38°, is in the neighbourhood of pH 9.4, though it is perhaps doubtful whether in the concentration of glycerol required to obtain satisfactory synthesis (50 per cent glycerol or above) the pH value has an interpretable significance. It suggests however that the optimum pH for synthesis is not widely removed from that required for hydrolysis.

It has recently been shown by Roche (1931) that, in presence of inorganic phosphate, *plasma* phosphatase will esterify glycol, glycerol,

glucose, fructose and glyceric acid under suitable conditions, and that the phosphatase of *red blood cells* will also esterify glycol and glycerol, but glucose and fructose to a much smaller extent.

It is thus proven that animal phosphatases (and among them the

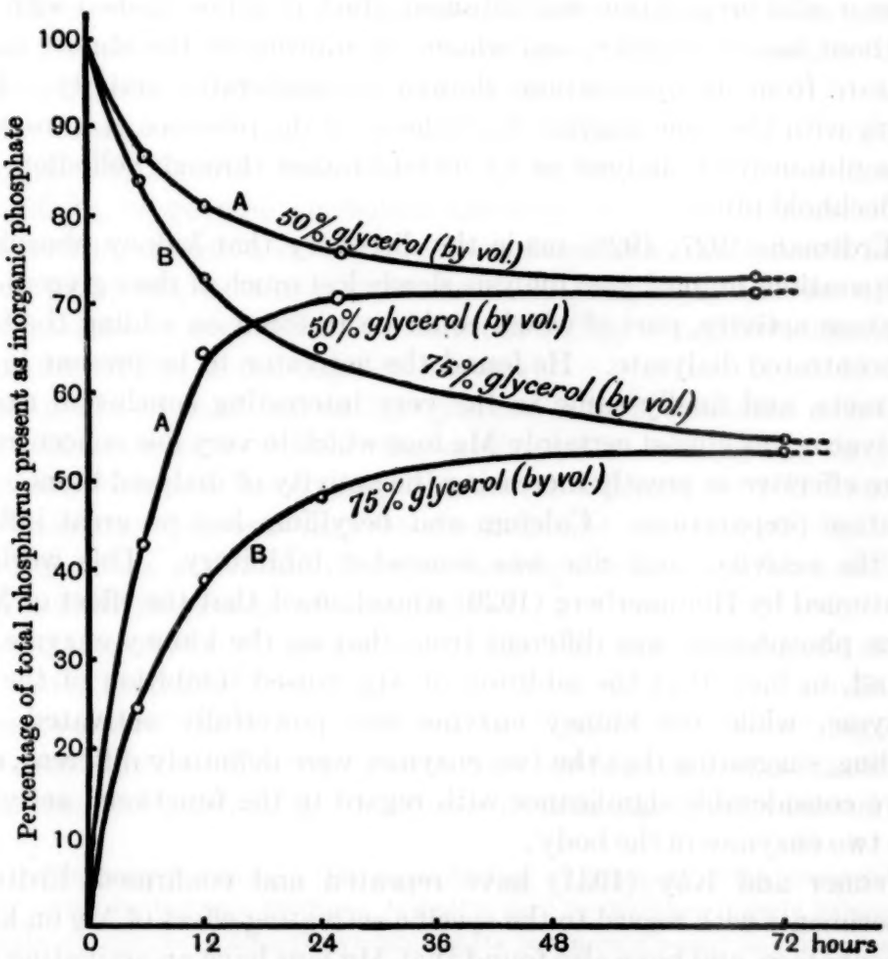


Fig. 2. Time relationships in the synthesis and hydrolysis of sodium glycerophosphate by duodenal phosphatase. 0 = pure Na β-glycerophosphate, 100 = pure Na_2HPO_4. Similar curves have been obtained with kidney and bone phosphatase.

bone enzyme) are capable of the synthesis as well as the hydrolysis of phosphoric esters, under conditions of concentrations of the reactants which it is conceivable might occur in specific localities within the tissues.

4. *A co-enzyme or activator.* Experiments with kidney phosphatase (Kay, 1926) and with the bone enzyme (Martland and Robison, 1929)

seemed at first to point to the absence of a dialysable co-enzyme for the animal phosphatases In the work with the kidney enzyme, simple dialysis against running water for 18 hours led to a 5 per cent loss of activity only, although the enzyme preparation had lost nearly all its inorganic salts and presumably practically all dialysable constituents. Also a solid preparation was obtained which could be washed with water without loss of activity, and which, on addition of the almost inactive filtrate from its preparation, showed no accelerated activity. In the work with the bone enzyme no evidence of the presence of a co-enzyme was obtained by dialysis or by ultrafiltration through collodion, using a Bechhold filter.

Erdtman (1927, 1928) made the discovery that kidney phosphatase preparations on *prolonged* dialysis slowly lost much of their glycero-phosphatase activity, part of which could be restored on adding the boiled, concentrated dialysate. He found the activator to be present in meat extracts, and finally came to the very interesting conclusion that the activator was almost certainly Mg ions which in very low concentrations were effective in greatly increasing the activity of dialysed kidney phosphatase preparations. Calcium and beryllium had no great influence on the activity, and zinc was somewhat inhibitory. This work was continued by Hommerberg (1929) who claimed that the effect of Mg on bone phosphatase was different from that on the kidney enzyme. He found, in fact, that the addition of Mg caused inhibition of the bone enzyme, while the kidney enzyme was powerfully activated. This finding, suggesting that the two enzymes were definitely different, might have considerable significance with regard to the functional activity of the two enzymes in the body.

Jenner and Kay (1931) have repeated and confirmed Erdtman's experiments with regard to the specific activating effect of Mg on kidney phosphatase, and have also found that Mg ions have an activating effect on all the animal phosphatases which have so far been tested, namely, those from kidney, intestinal mucosa, growing bone, adult bone, liver, red blood cells and blood plasma, either before or after dialysis. Dialysis slowly, and electrodialysis more rapidly, increases the percentage activation by Mg, though the activity tends to fall on prolonged dialysis. even after the addition of Mg. There is a suggestion that slow autolysis may play some part in the loss of activity on prolonged dialysis, possibly owing to slow liberation of otherwise indiffusible Mg from a colloidal complex and its consequent removal by dialysis. Thus with fresh preparations, although a short period of electrodialysis is very

effective in removing any inorganic salts which are in simple solution, it is less potent in effecting the inactivation of phosphatase (reversible by addition of Mg ions) than simple dialysis for some days against distilled water. No kation other than Mg shows the same constant and very large activating power. The same workers have found that *bone* enzyme is activated, in a manner entirely analogous with that of kidney phosphatase, by Mg in a wide range of concentrations, so that it seems possible that Hommerberg's contrary finding may require revision. Jenner and Kay have shown that Mg activates the enzymic hydrolysis of α- and β-glycerophosphates, of fermentation hexose diphosphate, of guanine nucleotide and even of pyrophosphate. The optimum concentration of Mg has been determined for several phosphatase preparations; it usually lies between q Mg 1.7 and 3.3 for different preparations under different conditions (q Mg = negative logarithm of molarity of Mg in the reaction mixture).

A dramatic effect of Mg on phosphatase is to be observed when red blood cell phosphatase of the dog or the rat is used as the enzyme. Under suitable conditions, using rat red cell enzyme, an activation of more than 3000 per cent may be shown in the presence of Mg. For some reason the activation of corresponding preparations from cells of the rabbit or the ox is much less marked.

The effects of Mg on the activity of dialysed phosphatase are of the same order as those of hydroxyl ions. To obtain optimal conditions (at least in vitro) for enzymic activity, both hydrogen ion and Mg concentration must be adjusted. As to whether Mg ion has or has not a similar effect on phosphatase in vivo we have as yet no information.

It is of interest to mention the recent finding of Lohmann, that the system in muscle which hydrolyses glycogen to lactic acid is dialysed muscle extract + inorganic phosphate + adenyl pyrophosphate (or muscle adenylic acid) + *a Mg salt*. In absence of any one of these components the system ceases to hydrolyse glycogen. Lohmann (1931) has also found that magnesium is necessary for the fermentative activity of the dialysed yeast-juice + co-zymase system or the dried yeast + co-zymase system. In the latter system the yeast must be washed with dilute acid (KH_2PO_4 solution) to remove the magnesium sufficiently. Euler, Nilsson and Auhagen (1931) agree that Mg is essential for the fermentation of glucose, fructose or sucrose by dried, washed yeast, but find that it is not essential for the activity of the redox system in this material.

c. *The Identity of the Phosphatases from Various Tissues.* If we consider any tissue extract, e.g., intestinal extract, which will hydrolyse a large number of P esters, there seems little reason to doubt that most,

if not all, of these esters are hydrolysed by one and the same enzyme. Experiments pointing in this direction are described by Kay (1928a) and by Levene and Dillon (1930). Even pyrophosphate is hydrolysed by various tissue extracts in such a way as to suggest the identity, in mammalian tissues at least, of pyrophosphatase and the orthophosphoric esterase (Kay, 1928b). It is, however, conceivable that certain substrates, such as hexose diphosphate may be hydrolysed by enzymes whose primary function is to break up the hexose, and which only liberate inorganic phosphate as a by-product, so that in certain tissues hexosephosphatase may be found to have different properties from the true phosphatase whose primary point of attack is the ester linkage.

Another question, bound up with that discussed in the preceding paragraph, is whether the phosphatase activity of the various active tissues of the body (bone, kidney, lung, intestine, etc.) is due to the same enzyme in every tissue, or whether there is a specific phosphatase for each or any of the tissues. It seems almost certain that it is the *same enzyme* which is present to a greater or lesser extent in most of the active tissues, with one possible exception, the phosphatase of red blood cells (Roche, 1931). The phosphatases from four tissues at least (bone, intestinal mucosa, kidney, blood plasma) have been investigated in sufficient detail to make out a good case for their identity. The evidence for this identity is as follows:

a. The same procedure, i.e., thorough grinding with chloroform water followed by a few hours' autolysis of the mixture at room temperature and filtration or centrifugation, is equally effective in the almost complete removal of the phosphatase from the different tissues.

b. The optimum pH for the phosphatase activity of the preparations from the four tissues is the same.

c Pyrophosphate, a somewhat anomalous phosphoric ester, is hydrolysed by each of the tissue extracts at a different optimum pH, pH 7 2 to 7.8, from that for the hydrolysis of the majority of phosphoric esters (pH 8.8–9.3), but the same optimum pyrophosphatase pH is shown by all the tissue extracts.

d. Mg stimulates the activity of all the four phosphatases, and the optimum q Mg is about the same for each of the four.

e. If a series of esters be hydrolysed the ratio of the rates of hydrolysis from one ester to another is the same with extracts of each of the four tissues.

f. The dissociation constant of the enzyme-substrate compound (Michaelis and Menten, 1913) is approximately the same for each of

the four tissue extracts acting on the same substrate—sodium β-glycerophosphate.

g. Finally kidney, bone, intestinal and plasma phosphatase will all *synthesise* phosphoric esters under suitable conditions, which conditions are similar for each of the tissue extracts. The above evidence is taken from Kay (1928a, b), Jenner and Kay (1931), Roche (1931) and unpublished experiments of the writer.

Phosphatase, therefore, is an enzyme present in animal tissues, not only in ossifying cartilage but in many other tissues also. It is particularly active in intestinal mucosa, and in the kidney cortex. It will hydrolyse practically every monophosphoric ester, both aliphatic and aromatic, which is presented to it, including a portion of the phosphoric esters of the circulating red cells, and the phosphoric esters present in relatively small amount in circulating plasma. Its activity, at least in vitro, shows an optimum at a hydrogen ion concentration of about pH 9, and at a Mg concentration of about 0.01 to 0.001 molar. Phosphatase belongs to the rather small group of enzymes which have been shown to catalyse under suitable conditions, the synthetic, as well as the hydrolytic, reaction.

THE BIOLOGICAL SIGNIFICANCE OF BONE PHOSPHATASE. The foregoing section has summarised most of the available information with regard to the properties of the animal phosphatases, and some idea will have been gained as to what bone phosphatase can and cannot do. In the light of these findings we are now in a better position to review the experimental data which have accumulated during the past eight or nine years and their bearing on the suggestion originally put forward by Robison that the phosphatase of ossifying cartilage is actively concerned in the deposition of calcium phosphate in growing bone.

This hypothesis (Robison and Soames, 1924) may be stated a little more fully as follows: the osteoblasts, the hypertrophic cartilage cells, and certain cells of the inner portion of the periosteum in a growing bone contain, or can secrete, a very active phosphatase, which by hydrolysing the salts of phosphoric esters brought to the ossifying zone by the blood stream, cause a *local* increase in the concentration of phosphate ions. The solubility product for calcium phosphate, either $CaHPO_4$, $Ca_3(PO_4)_2$ or some more basic compound, which is probably very nearly reached at the concentration of inorganic phosphate and ionised calcium normally present in the circulating plasma at normal plasma pH, is thus exceeded locally, and a deposition of the calcium phosphate is brought about in, or in the neighbourhood of, the cells which secrete

the active enzyme. It will be noticed that the theory does not stipulate any particular composition for the mineral salts of bone, other than the undoubted presence in this tissue of some relatively insoluble phosphate of calcium, nor does it require any particular phosphoric ester, other than one with a relatively soluble calcium salt, to be circulating in the blood.

For convenience of review the experimental findings will be divided, possibly rather arbitrarily, into sections.

a. *Location of the Bone Phosphatase. Calcification in vitro.* Robison (1923) had shown that the enzyme was abundantly present in ossifying cartilage, and in every young bone he examined, but it was not present in non-ossifying cartilage. Robison and Soames (1924) found that a high degree of phosphatase activity was shown by the teeth of young animals, where calcium phosphate deposition was also rapidly proceeding. They showed that the long bones of rachitic rats, despite their inferior calcification, still contained the enzyme in as large quantities as comparable normal bones. If a long bone, taken from a rachitic animal, were split lengthwise, and one-half immersed for a few hours in a solution of calcium hexosemonophosphate or glycerophosphate at 37° and pH 8.4 to 9.4, there followed the deposition of fresh calcium phosphate in the zone of provisional calcification, and particularly in the region of the hypertrophic cartilage cells, i.e., in the area where normal calcification should have taken place and where the first deposits of calcium phosphate would have appeared if the rachitic rat had been allowed to survive and had been given sufficient vitamin D. The control halves of the bones were immersed in solutions of sodium chloride, sodium phosphate and calcium chloride, and in each case gave negative results, no fresh deposition of calcium phosphate being demonstrable by the same staining method (von Kossa's silver nitrate method).

These early experiments of Robison and Soames received somewhat severe criticism from Shipley, Kramer and Howland (1926) who stated that they were able to obtain calcification of rachitic rat bones (Shipley, 1924) when slices of the latter were immersed either in sterile serum from normal (but not from rachitic) animals, or in inorganic salt solutions containing both calcium and inorganic phosphate in the concentrations found in blood serum, together with sodium bicarbonate, sodium chloride and a little magnesium sulphate, the solution being at plasma pH. The addition of a one per cent protein (egg albumin) to this salt solution did not completely inhibit calcification, but in greater concentrations of protein no calcification took place. This calcification they found to

be inhibited by antiseptics, and by heating or cooling the bone. In criticism of the enzyme theory of calcification they pointed out that the calcium and phosphorus of serum had not yet been shown to be present in significant amounts as calcium hexosemonophosphoric acid (Robison had suggested that the phosphoric ester concerned in calcification was a hexosephosphoric ester with a soluble calcium salt). They also found it difficult to understand 1, why, if the enzyme was abundantly present in rachitic bone, the latter did not calcify in vivo; 2, what function the enzyme was supposed to play in tissues such as the kidneys, which did not ossify. They were of opinion that the pH used by Robison and Soames (pH 8.4 or higher) was unphysiological, and considered that the deposition of calcium phosphate in split rachitic bones after immersion in calcium hexosemonophosphate or calcium glycerophosphate, as shown in the illustrations submitted by these workers, to be quite unlike true calcification. In reply Robison (1926) admitted that any phosphoric ester (with a soluble Ca salt) which was hydrolysable by the bone enzyme would serve, and that a *hexose* phosphoric ester was one possibility only. He stated that the failure of other factors than the bone enzyme would be sufficient to prevent the normal calcification process from taking place. He pointed out that, owing to the absence from their solutions of proteins and other organic constituents which are known greatly to depress the ionisation of calcium in plasma, the salt solutions used by Shipley, Kramer and Howland, were actually much higher in calcium *ion* concentration than plasma—perhaps three or four times as high, and that their results were therefore explicable on the basis of a precipitation of calcium phosphate from a highly supersaturated solution, which might be expected to take place primarily in the neighbourhood of the solid phase. He suggested as a supplementary possibility that the cells which secrete the enzyme in bone might also bring about a slight increase in the pH of the matrix fluid, thereby decreasing the solubility of calcium phosphate and carbonate in this fluid whilst at the same time increasing the activity of the enzyme. In this connection it may be remarked that both Klement (1931) and Morgulis (1931) believe that the main mineral constituent of bone is more basic than $Ca_3(PO_4)_2$, and formulate this substance as $6[Ca_3(PO_4)_2] \cdot Ca(OH)_2$.

With Soames (1930) Robison repeated some of the experiments of Shipley and collaborators, and found that whereas *in vitro* it was not possible to obtain calcification of rachitic bone from rats and rabbits on immersing slices of these bones in salt solutions with a [Ca] × [P] below 40, yet on the addition of very small amounts of phosphoric ester (gly-

cerophosphate) to such solutions, calcification of similar slices proceeded rapidly. The amount of organic P added (as glycerophosphate) was not much greater than the amount of phosphoric ester P in plasma (see p. 408). These solutions, however, contained no protein, and no other organic constituent but phosphoric ester. The concentration of Ca ions was, therefore higher than in plasma, although the *total* calcium might be the same.

Robison, Macleod and Rosenheim (1930) continued these experiments, and obtained results which "set in sharp distinction two mechanisms in calcifying hypertrophic cartilage, the phosphatase mechanism which can produce in the matrix a local supersaturation sufficiently great to cause deposition of the bone phosphate, and the second, still obscure, mechanism which favours and may determine deposition of the bone phosphate from its supersaturated solution." They investigated in the first place, the effect, on the *in vitro* calcification of rachitic bone slices, of changes in the composition of the fluid bathing the tissues. To their basal solution containing 0.2 g. $NaHCO_3$, 0.5 g. NaCl, 0.03 g. KCl and 0.02 g. $MgSO_4 \cdot 7H_2O$ per 100 cc., and kept at a pH of 7.2 to 7.4 by equilibration with air containing 5 per cent CO_2, various known amounts of Ca, inorganic phosphate and organic phosphate were added. The resulting solutions were allowed to flow at the same slow rate over similar slices of rachitic bone in an apparatus which permitted the calcifying power of twelve solutions of different composition to be tested simultaneously on twelve similar slices of bone.

The bone slices were placed individually in tubes through which the liquid to be tested was allowed to flow slowly, so that any extra inorganic phosphate produced in the medium by the action of phosphatase which had leaked out of the bone was removed as soon as formed. Thus the composition of the fluid in contact with the bone was that intended, and not actually one a little higher in inorganic phosphate. To allow the bone to remain in contact with a liquid whose phosphate ion concentration was slowly rising would most probably have led to error.

It was found that definitely higher [Ca] × [P] levels were necessary for calcification when the concentration of bicarbonate was reduced from 0.2 per cent to 0.03 per cent, and still higher levels were required when the bicarbonate was entirely omitted. The view of Barillé (1910), which is supported by Watt (1925) that a double salt, a complex carbono-phosphate, is the substance first precipitated in the calcifying cartilage may perhaps receive further support from this finding. It was also shown that in presence of phosphoric ester, calcification took

place at considerably lower [Ca] × [P] levels than in its absence, whilst at higher [Ca] × [P] levels the effect of the ester was seen in greatly increased extent and density of the deposit. Chloroform or cyanide strongly inhibited calcification in inorganic solutions *but had no appreciable effect in solutions containing phosphoric ester.* Bone slices after being soaked in acetone, or desiccated in vacuo over H_2SO_4 lost their ability to calcify when immersed subsequently in *inorganic* solutions of high [Ca] × [P] levels, but showed extensive calcification with apparently normal deposits after immersion in solutions containing phosphoric ester. Robison, Macleod and Rosenheim concluded that there were two distinct mechanisms by which hypertrophic cartilage could undergo calcification; *a*, the phosphatase mechanism, relatively resistant to antiseptics and desiccation, which produces in the matrix fluid a local condition of supersaturation with respect to the bone phosphate; *b*. the "inorganic" mechanism, much more readily destroyed by antiseptics and desiccation, which favours deposition of the bone phosphate from supersaturated solutions.

They found that an adverse effect on calcification, either in presence or in absence of small quantities of phosphoric ester, was shown by quite small concentrations of Mg ions (0.84 mM or q Mg 3.1). These results confirm previous findings of Kramer, Shelling and Orent (1927) and Shipley and Holt (1927) that magnesium in low concentrations specifically inhibits calcification in inorganic salt solutions in vitro. When the specific *activating* effect of Mg ions on bone phosphatase is remembered (p. 396), this observation is not without interest.

Phosphatase occurs in the periosteum in a long bone as well as in the epiphyseal region. If the periosteum torn from a rachitic rat's bone be immersed in a solution of calcium glycerophosphate of suitable pH, a deposit of freshly precipitated calcium phosphate may be detected by the usual methods of staining in the inner surface of the tissue (Robison and Soames, 1924).

The enzyme is present also in *membrane bones* as actively as in cartilage bones. This was first shown by Martland and Robison (1924) for an ossified fetal parietal, and has since been confirmed by Fell and Robison (1930) for the embryonic mandibles of developing chicks. As a control, the non-ossifying Meckel's cartilage from the same region in the chick was examined at the same time. It contained no phosphatase.

It has been repeatedly observed (Robison, 1923; Martland and Robison, 1924; Kay, 1926; Fell and Robison, 1929) that non-ossifying cartilage, or cartilage before any detectable calcification has begun, shows

no phosphatase activity. This has been shown for tracheal cartilage, rib cartilage away from the costochondral junction, the unossified cartilage of the fetal pelvis, the patella before ossification, the unossified epiphysis in the fetal femur, tibia and humerus, and the femur of fowl embryos before calcification.

b. *The Enzyme in Bone Development.* 1. *Embryonic bone.* The time relationships of the development of the enzyme in young bone provide further evidence in favour of its importance in the early stages of ossification. The enzyme does not appear in embryonic cartilage or cartilage about to ossify until the appearance of hypertrophic cartilage cells or centres of ossification. The first histological signs of ossification and the first chemically detectable appearance of phosphatase are almost simultaneous. An observation pointing in this direction was made by the present writer on material provided by Needham (1923). It was observed that whilst phosphatase appeared to be absent from the unincubated egg, and not present in more than minimal quantities in the embryo up to the 8th or 9th day, by the 12th day, by which time calcification of the bones was proceeding rapidly, it was present in the embryo in considerable quantity, and was particularly active in the bones. Moreover a substrate for this enzyme appeared to be completely absent from the unincubated egg, but appeared in the embryo almost simultaneously with the enzyme. This observation, though interesting, was very preliminary, and little more than qualitative. The same general conclusion was indicated much more clearly by the work of Martland and Robison (1924) on the human patella. This structure remains entirely cartilaginous until three years or more after birth, when a single centre of ossification appears. Cartilaginous patellae obtained post mortem contained no phosphatase; where ossification had just begun the partly ossified portion of the cartilage showed marked phosphatase activity, whereas the unossified portion was quite inactive.

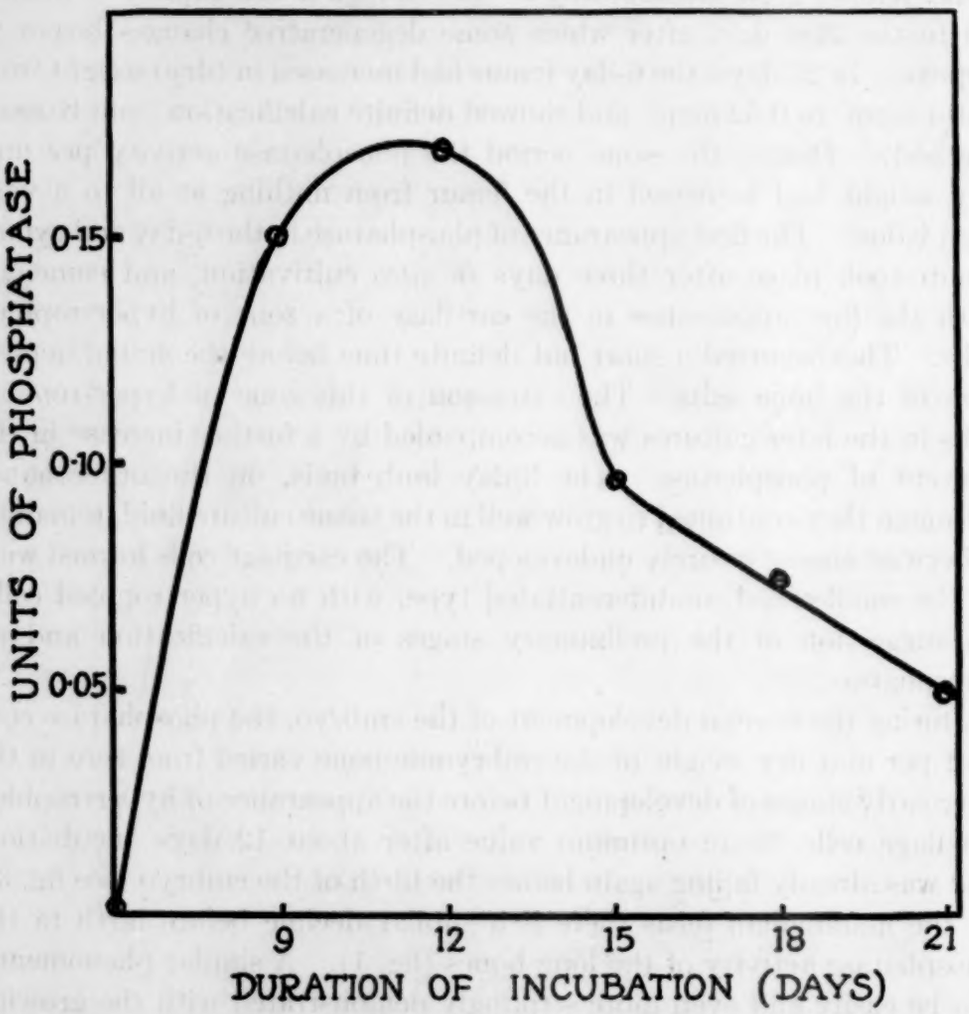
The very close correlation between the development in the growing embryo of the histological appearance characteristic of bone and the increasing phosphatase content of the same structure has been beautifully shown in recent work of Fell and Robison (1929), with isolated femora of young fowl embryos grown in tissue culture. Fell (1928) had previously shown that if terminal fragments of 8-day embryonic limb cartilage were explanted *in vitro*, they differentiated during subsequent cultivation into a diaphyseal portion containing hypertrophied cells and an epiphyseal portion containing smaller cells. In some cases *bone was deposited* around the diaphyseal portion.

In the 1929 work, femora (which are mainly made up, at this stage of development, of a very early type of cartilage cell with no trace of bone) were isolated from 5½- and 6-day fowl embryos and grown in a culture medium of plasma + embryo extract. The entire limb-buds (anterior and posterior) of 3-day embryos were similarly cultured. The 6-day femora grew satisfactorily *in vitro* though not as rapidly as *in vivo*, up to the 21st day, after which some degenerative changes began to appear. In 27 days, the 6-day femur had increased in (dry) weight from 0.014 mgm. to 0.52 mgm. and showed definite calcification (von Kossa's method). During the same period the phosphatase activity per unit dry weight had increased in the femur from nothing at all to a very high value. The first appearance of phosphatase in the 6-day embryonic femur took place after three days *in vitro* cultivation, and coincided with the first appearance in the cartilage of a zone of hypertrophied cells. This occurred a short but definite time before the actual deposition of the bone salts. The extension of this zone of hypertrophied cells in the later cultures was accompanied by a further increase in the content of phosphatase. The 3-day limb buds, on the other hand, although they continued to grow well in the tissue culture fluid, remained otherwise almost entirely undeveloped. The cartilage cells formed were of the small-celled, undifferentiated type, with no hypertrophied cells, no suggestion of the preliminary stages of the calcification and no phosphatase.

During the normal development of the embryo, the phosphatase content per unit dry weight of the embryonic bone varied from zero in the very early stages of development before the appearance of hypertrophied cartilage cells, to an optimum value after about 12 days' incubation, and was already falling again before the birth of the embryo (see fig. 3). In the mammalian fetus there is a similar decline before birth in the phosphatase activity of the long bones (fig. 1). A similar phenomenon can be easily and even more strikingly demonstrated with the growing incisors of rats, which are extremely minute at birth, and before birth can only be separated mechanically from the surrounding tissues with difficulty, but in which a very rapid decline in phosphatase per unit dry weight may be shown to take place post partum (fig. 1). On the other hand the phosphatase activity of the kidney in the fetus and in the young growing animal after birth shows the directly opposite trend. *In utero* it is relatively very low, and increases steadily and fairly rapidly after birth (fig. 1).

2. *Heterotopic bone.* Huggins (1931a) observed that when a portion

of the bladder epithelium of a dog was transplanted to the connective tissue of the abdominal wall, ossification regularly occurred after 18 to 20 days in the connective tissue, provided the epithelium survived. The bone so formed had a constant relationship to the epithelium; the epithelium itself did not ossify. When the bone was examined



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It has also been observed by Kay and Carscadden (1931) that in two samples of heterotopic bone removed from two patients the phosphatase content of the bone was of the same order as that for the rapidly growing bones of young rats, whereas control unossified tissues from the neighbourhood in which the bone was found contain very little of the enzyme.

c. *The Substrate for Bone Phosphatase.* As has been mentioned previously (p. 391) bone phosphatase will hydrolyse any phosphoric ester in which the phosphoric acid has one of its hydroxy groups esterified by almost any alcohol.

Assuming for the moment the truth of the phosphatase theory of calcification, it is necessary that a substrate containing phosphoric acid in ester combination should be conveyed to the growing bone, presumably by the blood stream. We are compelled to consider the *acid-soluble* phosphorus compounds of the blood, since the lipins (at least lecithin, kephalin and sphingomyelin) contain doubly bound phosphoric acid, and pure lecithin, at any rate, appears to be unhydrolysed by the bone enzyme (King, 1931), and it is unlikely, though not yet disproved, that the enzyme hydrolyses the other lipins.

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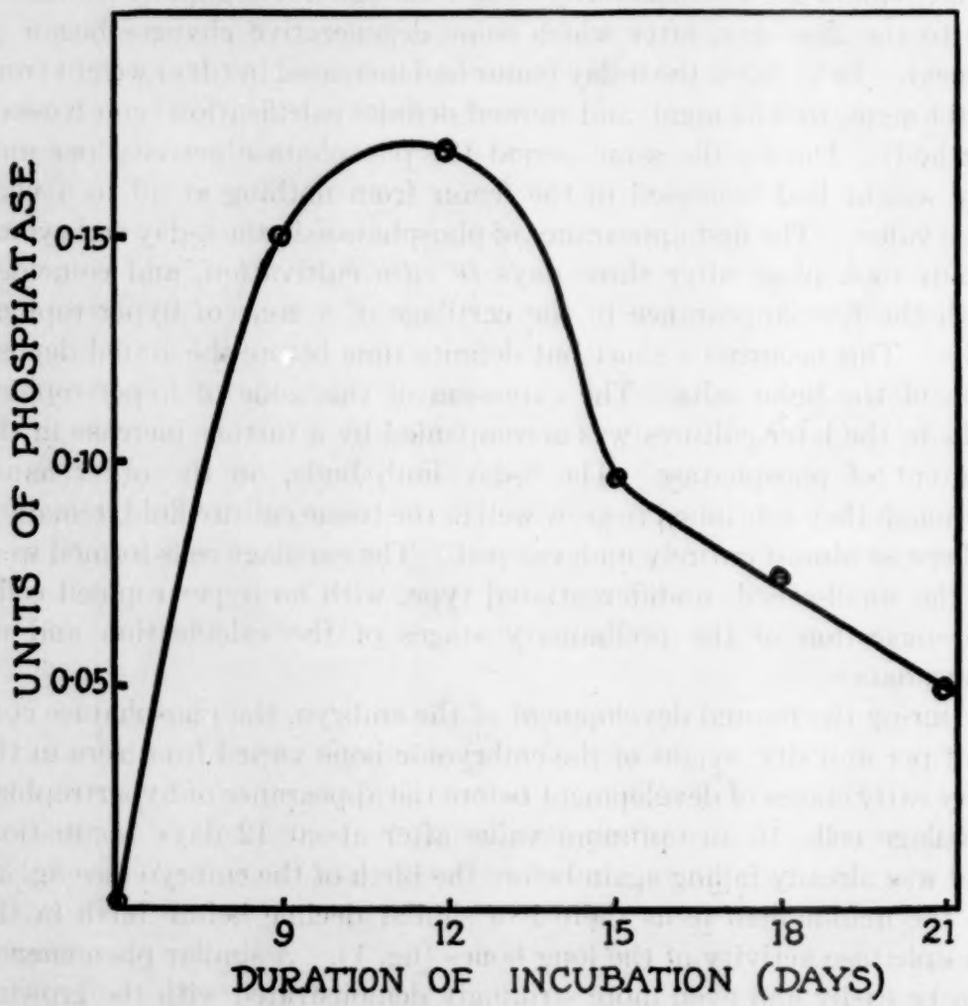

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Unfortunately, although a substrate is undoubtedly present *inside* the red cells, there is very little evidence that it can get out of the

unhemolysed cells without first undergoing hydrolysis, so that the question as to whether the phosphoric esters of the red cells can be the true, *in vivo* substrate for the bone enzyme remains unsettled. But it is not necessary to postulate that the red cell esters function in this way. There is, in the plasma also, a relatively minute amount of acid-soluble phosphoric ester, hydrolysable by the bone enzyme, and which in virtue of its position in the plasma has the maximum possible chance of coming in contact with the active cells in the calcifying cartilage. In quantity the plasma substrate varies, in adult plasma, around a mean value, expressed as P, of some 0.33 mgm. per 100 cc., i.e., about one-tenth of the average inorganic P value. Is this rather insignificant quantity of phosphoric ester, which appears to be hydrolysable by phosphatase, enough to provide adequate substrate for the bone enzyme?

From the work of Robison, Macleod and Rosenheim (1930) it appears that at critical concentrations of the inorganic radicles in their salt solutions, 0.5 mgm. P per 100 cc. of esterified phosphate is sufficient to determine calcification, this amount being similar to that found in the blood plasma in young rats or children.

In the young, growing animal, the phosphoric ester of the plasma may be markedly raised above the mean adult level. There is, however, a possible source of error in some of the earlier figures for plasma-phosphoric-ester, since it was not realised that in presence of trichloroacetic or other strong acid, glycerophosphoric acid (phosphoric ester) is liberated in appreciable quantities from the precipitated lipins even at room temperatures if filtration does not occur within a few minutes of the precipitation of the plasma colloids by the acid (Kay, 1931a). At present it can hardly be said definitely that there is more phosphoric ester in blood plasma from young than in that derived from old animals.

Grosser (1920) observed that if calcium glycerophosphate in aqueous solution was administered subcutaneously to rachitic children, marked calcium retention occurred. Korenchevsky and Carr (1925) in the course of a research on the effect of glycerophosphates and phosphates on the skeleton of growing rats on a rickets-producing diet, administered the same salt subcutaneously, and came to a similar conclusion, namely that such injections were followed by considerably increased calcification of the skeleton. Similar injections of sodium glycerophosphate produced in most cases a little improvement, but the effect was much less than with the calcium salt. The equivalent amount of sodium dihydrogen phosphate produced no definite change in the calcification. The results of these experiments fall fairly well in line with the phos-

phatase theory of calcification, though the data given by Korenchevsky and Carr would be more convincing if they had adjusted the pH of all the injected solutions to 7.4. A few experiments of Robison and Soames (1925) are in agreement with their findings. The latter workers found also that the concentration of phosphoric esters in the blood was below the normal in rats on McCollum's rachitogenic diet 3143. Since the phosphoric esters are almost entirely within the red cells this finding may merely have meant that the rats were anemic; no data are presented on this point. Whether the deficiency of calcification in rickets is or is not due to a shortage of phosphoric ester in the blood with the consequent deficiency of substrate for the bone enzyme still remains to be demonstrated. There is in any case, no shortage of phosphatase in the rachitic bone. Moreover, as pointed out by Leriche and Policard (1928), the precipitation of the bone salts depends less on the composition of the blood than on that of the interstitial lymph in actual contact with the pre-osseous framework. A material may be abundant in blood, but much less abundant in lymph and *vice versa.* We have little knowledge of the composition of this lymph, though changes in the chemical composition of the blood plasma must obviously be reflected in it.

d. *Other Evidence in Support of the Phosphatase Hypothesis.* 1. *Deposition of other metallic phosphates in bone.* It is evident that if some mechanism exists for producing and maintaining a relatively high local inorganic phosphate concentration in the epiphyseal and in the subperiosteal region in bone, we should expect that the other metals besides calcium when accidentally or experimentally administered would, if they formed phosphates which were insoluble at a neutral or slightly alkaline reaction, tend to be selectively precipitated and maintained as phosphates in the bones. Such deposition would naturally be limited to metals which were not immediately lethal. It has been shown that lead (Gusserow, 1861; Minot and Aub, 1924; Aub, Fairhall, Minot and Resnikoff, 1925) strontium (Stoelzner, 1908) lithium (Desgrez and Meunier, 1927) silver (Gettler, Rhoads and Weiss, 1927) and radium (Flinn and Seidlin, 1929) are selectively localised in the bones under these conditions. Fairhall and Shaw (1924) have produced evidence which suggests that the lead is present in bone as the insoluble tertiary phosphate $Pb_3(PO_4)_2$. Moreover the same mechanisms which remove calcium from the bones also remove lead, namely, the administration of substances such as ammonium chloride which will produce acidosis (Minot and Aub, 1924) or the giving of fairly large

doses of the parathyroid hormone (Hunter and Aub, 1926) or of irradiated ergosterol (Taylor and Weld, 1932). Flinn and Seidlin (1929) used parathormone treatment in an attempt to increase the elimination of radium from the bones of factory workers poisoned by radium compounds. These findings have added interest since Aub (1928) and others have obtained evidence to suggest that the primary effect of the hormone is probably on phosphorus metabolism, and that the heavy metal is liberated from the bone by this agent because it is present there as the phosphate. All of the above metals form phosphates very insoluble at body pH.

As support for the phosphatase hypothesis of bone formation this evidence is obviously very indirect. It would indicate at least that a physico-chemical mechanism is at work in the bone, a mechanism unable to distinguish between the toxic lead and the physiological calcium, but which precipitates either equally as the insoluble phosphate. This same mechanism which, according to our hypothesis, is the high local inorganic phosphate concentration produced by the phosphatase, would then maintain both the calcium and the lead in the bone in the insoluble state.

2. *Diminution of bone phosphatase in "hypervitaminosis D."* The toxic effects of the administration of excess of irradiated ergosterol are well known, and references need not be given here. Kreitmair and Hinzelmann (1928) observed that when the administration of sub-lethal doses is continued for a considerable period, there is decalcification of the bones involving the ribs, extremities, spine and skull. Baumgartner, King and Page (1929) found shortly afterward that in rabbits to which excess of irradiated ergosterol had been given, along with the usual symptoms and post-mortem findings of calcification of the arteries, kidneys, lungs, etc., there was also a very marked diminution in the total phosphatase content of the bones. The phosphatase activity was reduced to about one quarter of the normal value for control animals. With another sample of irradiated ergosterol which did not appear to bring about metastatic calcification, Page and Reside (1930) found little or no change in the phosphatase content of the tissues. Unless, as Heymann (1930) points out, Page and Reside were using a preparation which was absolutely inactive, their results would suggest that the diminution in phosphatase in the bones and the general upset in calcium-phosphorus equilibrium were associated phenomena, and that they could be interpreted as indicating the importance of the bone phosphatase in the maintenance of the *status quo* in adult bone.

Some unpublished work of Branion, King, Nicholson and Kay has confirmed the original findings of Baumgartner, King and Page. They have shown that both in rabbits and in rats the tissue phosphatases are markedly diminished after irradiated ergosterol feeding, the diminution being roughly proportional to the intensity of metastatic calcification.

Further experiments of Page (1930) have indicated the similar effect on the bone phosphatase of the administration of parathormone which, as just mentioned, will produce an active mobilisation of calcium and phosphorus from the skeleton (Bauer, Aub and Albright, 1929; Lambie, Kermak and Harvey, 1929). Injection of rather large quantities of parathormone (Lilly) was found by Page definitely to depress the activity of the bone enzyme in rats, though the effect is smaller than with irradiated ergosterol. Page claims this finding as confirmation of his view that the mobilisation of calcium and phosphate from the bones is a process in which the diminution in activity of the bone enzyme is one of the first steps.

3. *The phosphatase content of the blood plasma in bone disease.* It has recently been shown (Kay 1929b; 1930b) that in certain generalised diseases of bone, and almost without exception in no other disease, the plasma phosphatase—an enzyme having all the properties of the bone enzyme, and apparently identical with it—is markedly raised in quantity. Whilst in normals the phosphatase (expressed in arbitrary units) varies from 0.10 to 0.21, in cases of osteitis deformans, generalised osteitis fibrosa, osteomalacia and rickets (infantile, adolescent and renal) the value is much increased, rising in some cases to above 3 units which is more than 20 times the normal average value. The increase above the normal may be correlated in a general way with the severity of the disease. To the two constituents of the blood which have been known for some years to be affected to a greater or lesser extent in bone diseases, namely, the serum calcium and inorganic phosphate, must therefore be added a third variable, the plasma phosphatase, which suffers still greater changes.

What these changes are may be seen from table 3, which is taken mainly from the results of Kay (1930b), Hunter (1930, 1931) and unpublished results of the present writer.

In a few cases of generalised osteitis fibrosa with definite hyperparathyroidism phosphatase determinations have been made in the plasma before and after parathyroidectomy. In these cases there has been a slow fall in the phosphatase content of the plasma corresponding with increasing calcification of the bones as shown by x-ray (Hunter, 1930,

1931). The same phenomenon is also exhibited by a case of osteomalacia, not shown in table 3. In this case there was no operation, but the patient, aged 21, with a very unsatisfactory dietary history and distressing deformities, was given a diet rich in calcium, phosphorus and vitamins. Her plasma phosphatase changes were as follows: 1929, November, 1.8 units; 1930, January 6, 1.65; January 23, 1.0; March, 1.1; April, 0.51; July, 0.31; November, 0.22; December, 0.18; 1931, January,

TABLE 3

Changes in the phosphatase content of the plasma in disease

DISEASE	NUMBER OF CASES	PHOSPHATASE CONTENT OF PLASMA		
		Highest	Lowest	Mean
		units	*units*	*units*
None. Normals	33	0.21	0.08	0.14
Diabetes, mostly on insulin	50	0.55	0.11	0.26
Renal disease, excluding renal rickets and renal neoplasma	34	0.33	0.11	0.21
Arthritis without bony changes	11	0.33	0.11	0.17
Arthritis with bony changes	7	0.25	0.09	0.17
Exophthalmic goitre	7	0.75	0.27	0.47
	8*	0.53*	0.19*	0.36*
Osteomyelitis	8	0.41	0.14	0.27
Myositis ossificans	3	0.20	0.13	0.17
Fragilitas osseum (infants or children)	6	0.66	0.16	0.41
Acromegaly	2	0.32	0.22	0.27
Rickets (infantile)†	13	1.7	0.42	1.03
Rickets (renal)	2	1.5	0.9	1.2
Adolescent rickets	1			>2.4
Osteitis fibrosa (generalised)	3	>2.5	1.5	>1.8
	3*	1.8*	1.06*	>1.3*
Osteitis fibrosa (focal)	7*	0.25*	0.16*	0.21*
Osteitis deformans	24	3.4	0.65	>1.7

* Hunter (1930).

† Average for normal infants of approximately same age = 0.32.

0.14; December, 0.18; i.e., normal. Increasing bone density (x-ray) and clinical improvement followed the same course (Farquharson and Kay, 1932).

It will be observed from table 3 that the cases with generalised bone disease are in a quite different category from practically all other diseases, with exophthalmic goitre in an intermediate position. In many cases of the last disease there is a negative calcium balance, and the

bones not infrequently show evidence of widespread osteoporosis (Hunter, 1930).

The method of plasma phosphatase determination used in obtaining the results shown in table 3 is given in detail by Kay (1930a). This method calls for 48 hours' hydrolysis and requires 5 cc. of plasma. Another method has recently been worked out by Jenner and Kay (1932) which requires less expenditure of time (3 hours) and material (2 cc. of plasma or even less) and is in other ways more suited to the exigencies of the clinical laboratory.

4. *Some speculations.* The meaning of these changes in plasma phosphatase in disease is difficult to interpret. At the lowest estimate of their significance these findings establish another strong correlation between bone maintenance and phosphatase. The view, which is by no means new but perhaps not very general at present, that to obtain a better understanding of the biochemistry of bone formation, maintenance and pathology, emphasis should be laid perhaps more on the metabolism of phosphorus than on that of calcium is certainly supported by these findings.

In the next few paragraphs one or two small expeditions into realms of speculation, which may adumbrate certain directions in which information is required, will be undertaken.

It would be of great interest to ascertain whether in diseases such as osteitis deformans and generalised osteitis fibrosa there is, alongside the rise in plasma phosphatase, a diminution in bone phosphatase. The difficulties of such determinations with available clinical material are obvious and almost insuperable, but if it could be firmly established that experimental osteitis fibrosa produced in young guinea pigs and dogs by prolonged treatment with parathormone (Bodansky, Jaffe and Blair, 1930, 1931; Jaffe and Bodansky, 1930) is really comparable with the human disease, something might be done with this experimental material. It would also be of considerable interest to determine whether in regions of human bone where mal-union of fractures is particularly liable to occur, such as the neck of the femur or the junction of the middle and lower thirds of the tibia, there is abnormally low phosphatase in the sub-periosteal region.

The question arises as to whether the greatly increased phosphatase content of the plasma in bone disease is a cause or an effect of the disease. It might be established as a cause if it could be shown that owing to its increased activity in bone disease the plasma enzyme actually hydrolysed all or a considerable part of the circulating substrate for the bone

enzyme before the substrate arrived in the vicinity of the bone. Only a very few experiments have been carried out on this point, and are unconvincing (Kay, 1930b).

Byrom (1931) points out that if the essential abnormality in osteitis deformans were an increased plasma phosphatase which as far as the long bones were concerned starved the epiphysis and subperiosteal regions of the necessary substrate required for normal maintenance, one would expect the most marked changes to take place in these regions. This, however, does not appear to be the case. Moreover the evidence that generalised osteitis fibrosa is due to hyperparathyroidism seems to be quite conclusive, and it is hard to believe that in this condition *plasma* phosphatase can be of direct primary significance.

That the high plasma phosphatase should be a cause of bone disease is rendered still less likely by the fact that in widespread malignant disease of the bone where the bone lesion is of known origin, the plasma phosphatase is high. In such cases, as in generalised osteitis fibrosa, the rise in plasma phosphatase is almost certainly a secondary phenomenon.

If it is not a cause, and if its presence is not due to an accidental coincidence, then the rise in plasma phosphatase is presumably an effect, direct or indirect, of the bone disease. Does the enzyme leak out of the bone into the plasma in such disease or leak out at more than the normal rate? It may be that it is produced in excessive amounts in the bone as an attempt on the part of the organism at compensation for the lesion, or possibly, owing to weakness of the bone, there is a greater amount of bending and crushing which mechanically squeeze out some of the cell contents. In the case of generalised osteitis fibrosa the effect of the excessive activity of the parathyroid gland may be to change the permeability of the bone cells to the enzyme, and the apparent correlation between the rise in plasma phosphatase and in serum calcium in this disease may indicate a real physical connection between the two phenomena. The advent of a satisfactory method for the determination of the partition of calcium in the blood between ionised Ca, unionised but diffusible Ca, Ca held by protein, etc., would be of the greatest assistance in this regard. Though in view of the irregular distribution of the enzyme in the bone and kidney such a calculation may have little real meaning, it may be computed that the normal content of phosphatase in unit volume of plasma is of the order of one three-hundredths of that in the same amount of an average sample of adult human kidney tissue, and perhaps about one-hundredth of that in an equivalent

quantity of an average sample of adult human long bone. A high plasma phosphatase level may therefore not necessarily mean a continuous high rate of leakage of the enzyme from the bones into the blood, but a chronic slow diffusion thither at slightly above normal rates.

The phosphatase of bone has been shown in vitro to be capable of synthetic as well as hydrolytic activity. It may be that the enzyme plays an active part also in bone resorption and demineralisation, under both normal and pathological conditions, by synthesising from some of the insoluble calcium phosphate of the bone soluble phosphoric esters of calcium. It is also not impossible, though perhaps unlikely, that the synthetic as well as the hydrolytic activity of the enzyme, plays a part in normal bone formation, that the effect of the enzyme is diphasic, producing synthesis of phosphoric esters in one part of the ossifying zone, and hydrolysis, with deposition of calcium phosphate, in another. This might explain the *in vivo* deposition of calcium phosphates from purely inorganic salt solutions, first noticed by Shipley, which takes place in the zone of cartilage cells containing a high concentration of the enzyme, and also the observation that in rickets, in osteomalacia, and in other diseases of bones, fractures are repaired just as readily as in the metabolically normal individual (Peters, 1930).

There is a remote possibility, in view of the effect of Mg as a specific stimulant of bone phosphatase activity, that the Mg in bone plays some such rôle. Changes in the Mg: Ca ratio in bone disease have been reported. It is not altogether impossible that Mg may be found useful in therapy in certain types of bone disease. Euler and Rydbom (1931) have found, for example, that a small proportion of magnesium added to the McCollum rachitogenic diet has an antirachitic effect.

Finally we have the possibility, first mentioned by Wells (1914), that there may be another local factor which influences calcium phosphate deposition, in that certain of the cells of the bone—possibly the same cells which secrete the phosphatase—may also bring about a slight increase in the pH of the matrix fluid, which would not only increase the activity of the enzyme but accelerate the local precipitation of the phosphate and carbonate of calcium, and also of magnesium. In fact without some such mechanism the retention of magnesium phosphate in bone is difficult to explain.

5. *A note on the possible presence of $CaHPO_4$ in bone.* The recent work of Klement (1931) and of Shear and Kramer and collaborators (1928, 1929, 1930), on the chemical composition of bone provides considerable support for the view that the mineral matter of bone is in

equilibrium with the calcium and phosphorus of the plasma through an intermediate compound occurring in bone in relatively small amounts only. The presence of this compound is completely concealed from ordinary chemical analysis by the large excess of the main chemical constituent of bone (which according to Klement is "half-basic" calcium phosphate, 6 $Ca_3(PO_4)_2 \cdot Ca(OH)_2$ mixed with $CaCO_3$). Both Klement and Kramer and Shear suggest that $CaHPO_4$ is the first compound laid down in bone, in equilibrium with the salts of the blood plasma. The latter workers, however, find that even in normal young animals in whom calcification is proceeding vigorously, the Ca and inorganic P concentrations of blood plasma fall just a little short of complete saturation values for $CaHPO_4$. They are of opinion that one or more local factors are essential for the precipitation of this intermediate compound in the ossifying zone.

The proved presence of phosphatase in this zone appears to provide one, at least, of the factors required, and in fact fits so aptly into their suggested scheme that one is rather tempted by the esthetics of the situation to accept as established the rôle of $CaHPO_4$ as an intermediate compound in ossification. Nevertheless, in view of the meagre factual support for the $CaHPO_4$ theory, to succumb immediately to this temptation would be unwise.

e. Difficulties of the phosphatase hypothesis. The evidence which leads one to regard the enzyme phosphatase as playing an active part in bone formation and bone disease is circumstantial. The enzyme is present in bone in largest quantities when and where calcification is taking place. It is observed to be present in unusual quantities in plasma when bone is disorganised. But it *is* present in tissues which do not normally calcify, such as the kidney and the intestinal mucosa. In one tissue at least, that under certain conditions calcifies, namely, the arterial system of the rabbit poisoned with irradiated ergosterol, the deposition of calcium phosphate takes place in a tissue which seems to show no phosphatase activity whatever (Branion, Kay, King and Nicholson, 1931). Also, why, in spite of the fact that there is a large quantity of enzyme present in such tissue (Mai, 1928), are lime salts not deposited in the zone of hypertrophic cartilage cells in *rachitic* animals?

The answers to all these difficulties cannot as yet be given. Forrai (1923) has endeavoured to correlate the phosphatase content of active tissues with their embryological origin, and makes the interesting suggestion that tissues originating in different germ layers may have a

different enzymology. It is a short step from this to the idea that as the cells of the developing tissues become specialised they may also specialise their primitive enzymic equipment for particular functions. The cells responsible for calcification in bone, for example, may develop their phosphatase as a primarily hydrolytic mechanism to subserve the function discussed in this review, whereas in other tissues the phosphatase mechanism may be employed for synthetic purposes, e.g., the formation of nucleic acids, and other phosphoric esters in the cells (including incidentally the substrate for the bone enzyme). The phosphatase of the intestinal mucosa, together with that secreted in the succus entericus, undoubtedly effect the breakdown of phosphoric esters in the food, possibly to protect the body against the uncontrolled influx of phosphoric esters which might interfere with the normal skeletal equilibrium.

In the case of the phosphatase of the kidney it has been suggested by Eichholtz, Brull and Robison (1925) that the acid-soluble organic phosphorus compounds of the plasma may be the normal source of part, if not all, of the inorganic phosphate of the urine, the organic phosphorus undergoing hydrolysis in the cells of the kidney under the action of kidney phosphatase. This view has been criticised by Brain, Kay and Marshall (1928), and so far the phosphatase of the kidney remains without a demonstrated specific function. The kidney, however, is an organ which may calcify if the blood supply to it is interfered with (Remesov, 1926).

The absence of calcification in rickets in spite of the high content of enzyme in the zone of hypertrophic cartilage is relatively easily explained on the basis of the low inorganic phosphate content of the circulating blood. The bone enzyme is not able to raise the inorganic phosphate concentration in the cartilage from the low plasma value to that necessary for the local precipitation of calcium phosphate. Another possible alternative, not yet investigated, is that in rickets the phosphoric ester of the *plasma* may be reduced so that the substrate for the bone enzyme is partly lacking. A ferroconcrete building cannot be erected, no matter how satisfactory the tools or the workmen if either concrete or iron is in insufficient quantity. To quote Shear and Kramer (1930), "with a given state of affairs in the provisional zone of calcification, the occurrence of calcification will depend on the chemistry of the serum. On the other hand, with a given state of affairs in the chemistry of the serum the occurrence of calcification will depend on the local factors."

CONCLUSION

Among the factors which have to be taken into account in the study of calcification and bone formation from the biochemical standpoint are *a*, the calcium content of the blood plasma and the existence of this calcium in several forms—ionised, unionised, dialysable and undialysable; *b*, the phosphate content of the plasma; *c*, the pH of the plasma; *d*, the presence of normal or abnormal quantities of the parathyroid hormone; *e*, the activity of the thyroid gland; *f*, the intake of calcium and phosphorus in the food; *g*, the effective acidity or alkalinity of the food; *h*, the presence or absence of vitamin D in the food or in the body; *i*, the reaction of the intestinal contents.

The importance of every one of these factors has at one time or another been emphasised, but in almost every discussion arising out of such studies the necessity for some local condition in the calcifying tissue, favouring *local* deposition of calcium and phosphate, has been pointed out. Suggestions as to the nature of this local factor have not been lacking. The local deposition has been ascribed to the "specific vital activity" of the calcifying cartilage (recalling Molière's well-known "dormitive principle" of opium—see Bayliss, 1918), to the low metabolic rate in the area of deposition, to specific adsorption, to specific compound formation between calcium and cartilage protein, to the supersaturation of plasma with calcium phosphate with its consequent deposition when the fluid comes in contact with the solid phase (i.e., bone, although as Robison and Soames (1924) point out, this would not explain the beginning of ossification in embryonic cartilage) to a local high pH which diminishes the solubility of Ca in presence of phosphate and carbonate ions.

The presence of phosphatase in calcifying cartilage, which, unlike several of the alternatives just mentioned, is neither a wordy formula nor a vague supposition, but an experimentally verifiable fact, provides a local factor for which there has been an obvious need ever since the first attempt was made to understand the biochemistry of bone formation.

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THE NATURE OF VIRUSES

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Viruses are usually characterized by three negative properties, namely, invisibility by ordinary microscopic methods, failure to be retained by filters impervious to well-known bacteria, and inability to propagate themselves in the absence of susceptible cells. I prefer a positive characterization of the viruses, one emphasizing the intimate relation that exists between them and their host cells. The multiplication of viruses only in the presence of susceptible cells, their regeneration and production of disease in many instances in only one species of host, the marked stimulation and destruction of cells induced by their activity which on the one hand gives rise to tumors, such as Rous' sarcoma, and on the other to vesicular lesions, as fever blisters, the intracellular pathology frequently evidenced in virus diseases by inclusion bodies, and, finally, the lasting immunity that follows the majority of virus maladies, are essential phenomena that serve to stress the intimate type of parasitism encountered in working with these active agents. Such a characterization of viruses implies much, not only as concerns their biological nature which is still a moot question, but as regards their activities about which something is definitely known.

Data concerning the nature of viruses are sufficiently adequate in quantity but distinctly lacking in quality. According to reports, some of which have come from eminent investigators, most of these active agents have been seen and have been cultivated on lifeless media. If such statements are correct, viruses are autonomous living agents, and further discussion of their biological nature should deal with their place in the scale of living entities and their relation to other forms of life. Reports of work in this field are confusing, however, particularly to the uninitiated, and critical investigators are of the opinion that enough reliable data have not been acquired to establish the nature of the viruses. Inasmuch as this is a subject of fundamental biological importance, I shall review some of the recently accumulated data regarding the size, electrical charge, purification, spontaneous generation, adaptations, elementary bodies, metabolism, immunological phenomena, and

cultivation of viruses that might be of assistance in the elucidation of the origin and constitution of these peculiar incitants of disease.

SIZE. The size of minute particles may be determined in several ways, namely, by direct mensuration provided the objects are capable of resolution under the microscope; by filtration and ultrafiltration if the factors that influence the passage of the particles through pores of graded diameters are known and controlled; by diffusion, and, finally, by centrifugation. All of these methods have been employed in the study of the magnitude of viruses and the results obtained will be discussed.

It is generally accepted that an object less than 0.2 μ or 200 $\mu\mu$[1] in diameter is not capable of resolution under the microscope when ordinary light is used. Furthermore, it is understood that mordants and stains usually increase the magnitude of small particles. Some of the "larger" viruses, *e.g.*, those of fowl-pox (109), smallpox, vaccinia (122, 123), and rabies, are said to be just visible after treatment with certain mordants and dyes. Consequently, one is justified in concluding that most of the viruses have a diameter of less than 200 $\mu\mu$ and in an unstained state are not mensurable by means of ordinary light. Moreover, figures regarding their size derived from stained preparations are apt to be inaccurate and misleading. The use of light of short wave lengths makes possible the mensuration of particles smaller than 0.2 μ in diameter. So far, however, this method of investigation has yielded no convincing evidence concerning the magnitude of viruses. It appears, therefore, that direct methods of mensuration only indicate that the active agents are considerably smaller than ordinary bacteria.

The sizes of at least eight viruses have been estimated by means of ultrafiltration, diffusion, or centrifugation. The results obtained for these active agents together with figures for the diameter of the hemoglobin molecule for comparison are given below.

Hemoglobin. For a number of years the molecule of hemoglobin was thought to be 30 $\mu\mu$ in diameter. Recently, however, figures (34) derived from the results of Svedberg and Nichol's (33) centrifugation experiments and Northrop and Anson's (30) diffusion experiments with hemoglobin indicate that its diameter is approximately 5.5 $\mu\mu$. Many estimations regarding the magnitude of viruses have been based on the former figure for the diameter of the hemoglobin molecule, 30 $\mu\mu$. If this figure is incorrect, many statements concerning the size of viruses are also inaccurate.

Mosaic virus. Duggar and Karrer (17) by means of ultrafiltration found the

[1] In this paper the symbol $\mu\mu$ represents 10^{-6} mm., a millimicron.

infectious particles of mosaic virus to be of the same order of magnitude as hemoglobin molecules, namely, 30 $\mu\mu$ in diameter. Vinson (34), however, says that Duggar's experiments interpreted in the light of recent work regarding the size of hemoglobin molecules indicates that the diameter of mosaic virus is about 5.5 $\mu\mu$.

Herpetic virus. Zinsser and Tang (38) by means of ultrafiltration estimated the diameter of herpetic virus to be 20–100 $\mu\mu$. Levaditi and Nicolau (27) in the same way found that the virus passed through membranes which retained toxins, hemolysins, complement, and serum globulins. Bedson (13), however, unable to confirm Levaditi and Nicolau's (27) results, obtained evidence by centrifugation that herpetic virus is probably of sufficient size to be visible.

Foot-and-mouth disease virus. Olitsky and Boëz (31), using ultrafiltration, found that the virus of foot-and-mouth disease is 20–100 $\mu\mu$ in diameter. Elford[2] by means of his special membranes estimated it to be 8–12 $\mu\mu$.

Poliomyelitic virus. By means of ultrafiltration, Krueger and Schultz (25), in 1929, found that the virus of poliomyelitis possesses a magnitude not greater than 300 $\mu\mu$. In 1931, by the same means, Clifton, Schultz, and Gebhardt (16) obtained results indicating that the diameter of the virus lies below 50 $\mu\mu$.

Fowl plague virus. By means of ultrafiltration Andriewsky (8) secured a figure of 2.5 $\mu\mu$ for the diameter of fowl plague virus, while Bechhold and Schlesinger (11) by centrifugation found it to be 120–130 $\mu\mu$.

Bacteriophage. According to d'Herelle (22) and Elford (19), both of whom used ultrafiltration, the diameter of the bacteriophage is approximately 30 $\mu\mu$. Kruger and Tamada (26) by means of purified bacteriophage preparations and ultrafiltration found it to be 5 $\mu\mu$, and Hetler and Bronfenbrenner (24) by means of a diffusion method estimated it to be 1.2–22.8 $\mu\mu$.

Rous virus. According to Zinsser and Tang (38), the Rous virus is 20–100 $\mu\mu$; according to Mendelsohn, Clifton and Lewis (29), 50 $\mu\mu$; according to Fränkel (20), 10 $\mu\mu$. All these workers obtained their figures by means of ultrafiltration.

Vaccine virus. Levaditi and Nicolau (27) reported that vaccine virus passes through membranes impervious to toxins, hemolysins, complement, and serum globulins. Bland (15), however, from the results of his centrifugation experiments not only concluded that Levaditi and Nicolau's findings are incorrect but that vaccine virus is probably large enough to be seen. Bechhold and Schlesinger (11) by means of centrifugation estimated that the active agent is 210–230 $\mu\mu$ in diameter, while Yaoi and Kasai (37) working with "purified" virus found that it diffused at the rate of fuchsin particles and is, therefore, not capable of being seen.

One cannot consider the results cited above without being amused and dismayed. Many of the workers seemed in no way concerned about the possibility that they might have been estimating not the magnitude of viruses, but the size of particles of degraded cells to which the viruses were attached. Other investigators, cognizant of the diffi-

[2] Report made before the Physiological Section of the British Association for the Advancement of Science, September 28, 1931.

culties of the problem, attempted to remove the viruses from such carriers. They were unable, however, to be assured that they had been successful and that they had obtained the correct figures for the size of the different viruses.

From the results of indirect methods of mensuration it is safe to conclude that viruses are small and that some of them may be exceedingly minute. If the figure of 210 $\mu\mu$ for the diameter of vaccine virus is accurate, there is no reason as far as size is concerned to suppose that the virus is not a living organism. On the other hand, if the figures of 1.2 $\mu\mu$, 5.5 $\mu\mu$, and 8 $\mu\mu$ for the bacteriophage, mosaic virus, and foot-and-mouth disease virus, respectively, are correct, it is obvious that these agents cannot be highly organised, because it is impossible that with such a magnitude they can consist of more than one, or at most several, molecules of protein. Unfortunately, none of the figures can be accepted without reservations. At present the exact size of no virus is known.

The numerous discordant results encountered in the literature dealing with the filterability, size, and visibility of viruses are probably due to inadequate experimentation, careless thinking, prejudice, imperfect experimental methods, and the difficult nature of the problems. One of the great needs at present is improvement in methods of microscopy, filtration, and purification of viruses in order that results obtained will approximate the true size of viruses and not the size of particles of other sorts on which the agents are adsorbed. It must be remembered, however, that the determination of the size of one virus will not establish the magnitude of another, because no more uniformity of dimensions should be expected among these agents than is found among bacteria and protozoa. Furthermore, it is not possible to derive proof of the animate or inanimate nature of viruses even from a correct estimation of their diameters, for, within limits as yet undetermined, life and death are not functions of size.

ELECTRICAL CHARGE. Most bacteria and proteins under ordinary biological conditions of hydrogen ion concentration carry a negative electrical charge.[3] When the nature of the viruses became a question of interest, attempts were made to ascertain their behavior in an electrical field in order that it might be compared with the action of proteins and bacteria under similar circumstances. Some of the results obtained with nine viruses are stated below.

[3] The pH range over which they are electronegatively charged depends on their isoelectric point.

Bacteriophage. Kligler and his co-workers (41) using a so-called "protein-free" bacteriophage found that the active agent was amphoteric in acid and decidedly alkaline solutions and chiefly negatively charged in neutral and mildly alkaline solutions. Krueger and his associates (42) stated that the bacteriophage is negatively charged between the hydrogen ion concentrations of 9.0–3.4, and positively charged at pH 3.35. Todd (48) found that the active agent carried a negative charge between the hydrogen ion concentrations of 3.36–7.6. The results of Natarajan and Hyde's (43) experiments indicate (1) that bacteriophages for typhoid bacilli and Flexner's dysentery bacilli are only electronegative between pH 4.9–9.3 and 5.4–9.3 respectively, (2) that small plaque coliphage is electronegative below pH 8.3, but with greater alkalinity moves to both poles, and (3) that large plaque coliphage is electronegative over a range of pH 5.4–6.1, while at a higher alkalinity it wanders to both poles.

Rabic virus. According to Glusman (40) and his associates fixed rabic virus is negatively charged over a range of pH 6.0–9.3.

Vaccine virus. Douglas and Smith (39) found that vaccine virus carried a negative charge between the hydrogen ion concentrations of 5.5–8.4. The experiments of Yaoi and Kasai (49) revealed that between pH 6–7 more virus collected at the positive than at the negative pole, and between pH 8–9 the active agent was demonstrable only at the anode.

Fowl-pox virus. Kligler and his co-workers (41) found fowl-pox virus in "protein-free" preparations to be positively charged on the acid side, amphoteric in neutral solutions, and negatively charged in alkaline solutions. According to Natarajan and Hyde (43), the active agent is amphoteric over a range of pH 6.4–9.3.

Foot-and-mouth disease virus. Olitsky and Boëz (44) believe that the virus of foot-and-mouth disease is positively charged, while Sichert-Modrow (47) is of the opinion that the active agent carries a negative charge over a range of pH 7.0–8.1.

Pcliomyelitic virus. According to Olitsky, Rhoads, and Long (45) poliomyelitic virus wanders to the anode.

Rous virus. Pulcher (46) found that the Rous virus was adsorbed on electropositive and not on electronegative hemoglobins and concluded that the active agent is negatively charged.

Virus of infectious myxomatosis of rabbits. According to Natarajan and Hyde (43), the virus of infectious myxomatosis of rabbits is electronegative over a range of pH 4.9–9.3.

Herpetic virus. Natarajan and Hyde (43) found herpetic virus to be electronegatively charged only between the hydrogen ion concentrations of 7.0–8.9.

From the results of the work cited above it is obvious that most workers have found that under ordinary biological conditions of hydrogen ion concentration certain viruses in an electrical field wander to the anode. Moreover, many investigators have stated that the viruses under these conditions are negatively charged and in this respect are similar to bacteria, cells, and numerous proteins. Others, however,

aware of the fact that virus preparations usually consist principally of proteins and bits of degraded cells from the host, realize that the electrical charges determined might not be those of the virus particles themselves but of their carriers, *i.e.*, material on which the virus particles are adsorbed. It is true that a few experiments have been performed with "protein-free" preparations of viruses. But an examination of the methods of purification fails to convince one that such purified viruses had been completely separated from their carriers. Therefore, at present it is impossible to state definitely what electrical charge is carried by the viruses.

PURIFICATION. Inasmuch as virus-containing emulsions consist chiefly of substances unrelated to the active agents themselves, it is natural that workers should attempt to obtain the viruses either in a pure or in a relatively pure state. Moreover, it is being realized that purified viruses are essential for the proper study of problems in this field, such as the estimation of the size of viruses, the determination of their electrical charge, and the investigation of immunological responses excited by them. In addition to the fact that purified viruses are of practical value, it is obvious that such preparations will also be of value to investigators interested in the theoretical problem of the nature of viruses. Indeed, Murphy (52, 55) has already concluded from the results of his experiments on the purification of the Rous agent that this disease-incitant is neither a virus nor a living organism. He states, "It is hardly conceivable that the active fraction which I have thus succeeded in obtaining, a substance purified by repeated precipitations, could carry with it through all these manipulations any living organism or virus. To me the enzyme-like nature of the principle seems to have been conclusively established. . . . ." However, most workers do not believe that Murphy is justified in concluding from the results of such experiments that the Rous agent is not a virus, because at least eight other viruses, *e.g.*, the incitants of infectious myxomatosis of rabbits (58), foot-and-mouth disease (64), bacteriophagy (50, 56, 57), fowl-pox (56), vaccinia (66, 68), rabies (66), poliomyelitis (65), and mosaic disease (67) have been subjected to manipulations similar to those used by Murphy and have been obtained, still active, in various states of purity.

Most methods of purification of viruses are based on the principles of precipitation by a variety of chemicals and selective adsorption and elution as used extensively in enzyme work. As yet, it is unlikely that a virus has been obtained in a state of absolute purity. Nevertheless,

the results already secured are encouraging and should excite further investigations. It may be possible in this way to attain eventually a more accurate concept of the nature of some viruses. For instance, it may be shown that in certain purified virus preparations the number of nitrogen atoms for each infectious unit or particle is insufficient to warrant the supposition that the agents are living, organized structures. Krueger and Tamada (57) have already suggested this viewpoint.

CULTIVATION. In the literature of twenty years ago it is not uncommon to encounter reports in which it was claimed that viruses had been successfully cultivated on lifeless media. These reports have not been confirmed and at present such claims are rarely made. A few, however, have been made in recent years. Frosch and Dahmen (78) stated that they were able to cultivate the virus of foot-and-mouth disease on ordinary media. But the German, English, and American Foot-and-Mouth Disease Commissions were unable to confirm their work. Olitsky (91) reported the cultivation of mosaic virus in a cell-free medium. Nevertheless, upon repeating his work he (92) has been forced to conclude that true multiplication of the virus was not obtained. Recently, Eagles and McClean (75, 76) reported that vaccine virus is capable of regeneration in a cell-free medium. A careful examination of their papers, however, leaves one in doubt as to whether some of their media were cell-free, and as to whether multiplication of the virus occurred in the nutrient materials that undoubtedly contained no cells. In my laboratory (86, 90, 93) during the last four years, Haagen, Muckenfuss, Li, and I have made numerous attempts to cultivate vaccine virus in cell-free media, many of which were similar to if not identical with those employed by Eagles and McClean. None of our efforts was successful. On the other hand, the cultivation of vaccine virus in the presence of cells surviving *in vitro* has been more consistently successful in our hands and in Maitland's (88) than it has been in Eagles and McClean's.

Although the cultivation of viruses in lifeless media has not been accomplished, it is generally conceded that these agents are capable of pullulation in the presence of susceptible cells either surviving or growing *in vitro*. The viruses of Rous' sarcoma (72), Virus III infection of rabbits (69), herpes febrilis (70), fowl-pox (77), vaccinia (79, 80, 86, 88), rabies (94), foot-and-mouth disease (83, 84, 85, 89), vesicular stomatitis (73), infectious myxomatosis of rabbits (71, 81), fowl plague (82), and probably the agents causing common colds (74) and poliomyelitis (87), have been cultivated in the presence of tissues surviving *in vitro*.

Moreover, the characteristic of species specificity possessed by many viruses is frequently reflected in their *in vitro* cultivation. For instance, fowl-pox virus (77), innocuous for mice and rats, does not regenerate in cultures of their tissues. Foot-and-mouth disease does not attack chickens and the virus (89) does not grow in cultures consisting of minced chick embryo and plasma. In addition to a species specificity, some viruses exhibit in cultivation experiments a predilection for certain kinds of cells. Fowl plague virus (82) multiplies in the presence of chick embryo skin and brain, but does not regenerate in pure cultures of fibroblasts. Foot-and-mouth disease virus (85) increases in amount when the culture medium contains minced guinea-pig embryo, but does not grow when fibroblasts or bits of heart muscle alone are present. Thus it appears that many viruses are capable of multiplication in tissue cultures and frequently retain under such conditions their species and cellular specificity. Nevertheless, it will be interesting to observe the results of further attempts to circumvent this species and cellular specificity of viruses by *in vitro* methods of cultivation.

A crucial experiment, if there be one, to decide the question of the autonomy of the viruses is their undisputed cultivation on lifeless media. It may be impossible, however, to accomplish such an experiment with all of the viruses, because some of them may be obligate parasites, as is the malarial organism. Thus in the quest for proof of the nature of viruses, we may find that many of them are invisible and incapable of regeneration in the absence of living susceptible host cells. Such a state of affairs will prevent, for a time at least, a complete definition of the nature of these peculiar incitants of disease. Nevertheless, we should obtain all the facts and make the most of them in the study of biological phenomena and in the better understanding and control of disease. For this purpose it is not absolutely essential to see and to cultivate the viruses on simple media any more than it is imperative to see and to know what electricity is in order to study the phenomena produced by it and to control its activity for our daily needs.

Metabolism. Much of the discussion concerning the nature of viruses has centered around the question as to whether they are animate or inanimate. In this relation, one would like to know what the evidence is regarding independent metabolic activities of these active agents. Technical difficulties have hindered this type of experimentation with viruses. Nevertheless, a few investigations (95–99) have been made, the results of which were negative. One must not conclude from such negative results, however, that viruses do not possess an

independent metabolism and are, therefore, inanimate substances, because the methods used for the detection of the metabolic activities may not have been sufficiently delicate.

ADAPTATION. Certain viruses inoculated into new hosts apparently undergo changes in some of their characteristics. Smallpox virus (100, 101) passed through monkeys to rabbits and calves and then back to man is no longer smallpox virus but vaccine virus, and the disease, vaccinia, caused by it is not contagious as is smallpox. The incitant of yellow fever (106, 107) passed through a large number of mice by means of intracerebral inoculations loses much of its pathogenicity for monkeys when inoculated intravenously or intraperitoneally, but gains the power of producing a transmissible encephalitis in monkeys receiving the inoculum in the brain. Such phenomena are spoken of as adaptations of viruses to new hosts, and, inasmuch as adaptation is considered a characteristic of living rather than lifeless material, they have been cited by some investigators (103) as proof of the animate nature of the viruses. On the other hand, workers, who believe that viruses are products of cellular perversion, state that the changes observed in the characteristics of the active agents when they are inoculated into alien hosts are to be expected, inasmuch as mouse, rabbit, monkey, and human cells, because of intrinsic differences, may not always manufacture identical substances as the result of similar stimuli. Therefore, they contend that the changes and adaptations are not accomplished by the agents themselves but by their hosts and, consequently, are not admissible as proof of the living nature of the viruses.

INCLUSIONS. Within the nucleus and cytoplasm of cells injured by viruses, certain peculiar structures, inclusion bodies, are frequently observed. Although many of these bodies are of importance in diagnostic and experimental work, numerous opinions exist concerning their nature. Lipschütz believes that the inclusions in many diseases (119) consist of compact masses of virus particles, yet he is of the opinion that such structures in measles (120) are nothing more than altered central bodies. Goodpasture (113) thinks that Negri bodies in rabies are composed of degenerated mitochondria and neurofibrils, while Levaditi (118) and Manouélian (121) consider them protozoa and designate them, respectively, *Glugea lyssae* and *Encephalitozoon rabiei*. Goodpasture and his associates (124, 125) have demonstrated that the incitant of fowl-pox is intimately associated with the Bollinger bodies which are made up of a lipoid capsule within which numerous small coccoid bodies are embedded in a protein matrix. On the other hand,

Glaser (112) has presented evidence that the polyhedral bodies, the characteristic inclusions in wilt diseases of caterpillars, consist of noninfectious crystalline protein. Thus, it appears that inclusions may arise in a number of ways and that they may or may not contain virus. Consequently, generalizations regarding these peculiar structures are hazardous at present.

The small coccoid bodies found in fowl-pox by Borrel (109) and in vaccinia by Paschen (122, 123) appear to be extremely minute organisms. In fact, one is justified in asking why these bodies are not convincing evidence of the organismal nature of certain viruses. The first reason is that one cannot by morphological and tinctorial data alone determine whether autonomous life exists in such small objects. Another reason is the fact that Goodpasture, while holding the belief that the small coccoid bodies in fowl-pox (124, 125) represent the virus, stated that similar structures, seen in rabic brains (113) and considered of etiological importance by Babes (108) and Koch (114–116), are probably degenerated mitochondria. Moreover, Borrel (110) has described similar bodies in other virus diseases the etiological agents of which have been shown by ultrafiltration to be incapable of resolution by microscopic methods. Furthermore, Craciun and Oppenheimer (111), who cultivated the small bodies of vaccinia and showed that they are closely associated with the virus, made the following statement, "We have from these studies no morphological proof of an increase in the number of granules, since they cannot readily be distinguished from other granules normally seen in tissue cultures." Finally, mitochondria in some respects resemble bacteria. They may decrease or increase numerically within cells, and their size and shape may be altered by appropriate stimuli. At times, they actually divide. Nevertheless, mitochondria are not considered autonomous living agents. Consequently, so far as I am aware, there is no convincing evidence—the specific agglutination of virus elementary bodies (184) by antiviral sera will be discussed later—to invalidate the conception that cells under the stimulus of viruses may react by the formation of numerous small coccoid bodies uniform in size and intimately associated with the stimulating agents. One would not consider such bodies microorganisms or hold that they consist of virus alone. Therefore, in spite of definite proof that viruses are present in certain types of inclusions, doubt still exists regarding the organismal nature of the small coccoid bodies found within them.

Other features observed in pathological processes induced by viruses,

e.g., hyperplasia and necrosis, are fully as important as are the inclusion bodies. The excessive stimulation of cells seen in some virus diseases, *e.g.*, fowl-pox and warts, leads one by analogy to think of malignant neoplasms. Undoubtedly a number of fowl tumors are caused by agents separable from cells, and, although there is no proof that mammalian tumors arise in this way, the possibility is worthy of consideration and offers an attractive field for work. The fact, however, that some tumors are produced by filterable agents is by no means conclusive evidence that all neoplasms (217) arise through the activity of such incitants.

VIRUSES AS FILTERABLE FORMS OF BACTERIA. For a long time a few investigators have held that certain virus diseases are induced by ordinary bacteria. Now that attention is being focused on filterable forms of bacteria, workers in increasing numbers (128, 131, 132, 134, 135) are adopting the belief that viruses are merely filterable, invisible, and noncultivable elements of ordinary bacteria. It has been claimed, and evidence of a kind has been offered to substantiate the assertions, that the bacteriophage (165, 166) is a form in the life cycle of lysogenic bacteria, that the viruses of yellow fever (131, 134, 135) and hog cholera (134, 135) are invisible forms of *Leptospira icteroides* and *B. suipestifer* respectively, that the etiological agent of scarlet fever (134, 135) is a filterable form of hemolytic streptococci, and that the incitants of poliomyelitis, epidemic encephalitis, fox encephalitis, common colds, measles, and influenza represent certain stages in the life cycle of green streptococci (131). Without going into details of the available knowledge of bacterial life cycles and their invisible and noncultivable forms, one can say that proof of many of the claims regarding them is lacking. In fact, if certain reports are correct, some of the filterable forms of bacteria are much smaller than are many of the viruses. Kendall (131) recently stated that "egg white, filtered through Berkefeld W filters (after dilution with sterile physiological saline solution) is rarely sterile." Such a statement raises embarrassing questions for workers in the virus field because many viruses will not pass through W filters. Since the existence of bacterial life cycles is doubtful, it seems unwarrantable to offer the presumptive filterable forms of them as evidence upon another unsolved problem, the nature of the viruses.

EFFECT OF PHYSICAL AND CHEMICAL AGENTS ON VIRUSES. Many years ago it was discovered that bile and saponin are injurious to protozoa but with a few exceptions are innocuous for bacteria. Consequently, when the question of the nature of viruses began to attract

attention, tests were made to determine what effect bile and saponin have on these incitants of disease. Many viruses, *e.g.*, rabic virus (141, 144), were found to be inactivated and because of this fact certain workers concluded that they are protozoa. Sufficient exceptions, however, have been encountered to invalidate the test as a means either of separating bacteria from protozoa or of defining the nature of viruses. The agent causing Rous' sarcoma (140) is more resistant to ultraviolet light than are bacteria, and Murphy (220) considers this fact as evidence in favor of his hypothesis of the enzyme-like nature of the virus. On the other hand, bacteriophage (139), the living nature of which many doubt, is just as sensitive to ultraviolet light as are bacteria. Sanderson (153), using a temperature of −78°C., found no diminution in the titer of two strains of bacteriophage subjected to 20 successive freezings and thawings. Since bacteria and cells are killed by repeated freezing and thawing, he concluded that bacteriophage must be something other than a living organism. Rivers (151) showed, however, that colon bacilli, Virus III, vaccine virus, herpetic virus, bacteriophage, complement, and trypsin are all either killed or inactivated by repeated freezing (−185°C.) and thawing and that, as might be expected, some of the agents are more resistant than are others. Hence it is obvious that destruction or inactivation of an active agent by repeated freezing and thawing is not evidence that it possesses life. The observations on heat, desiccation, oxidation, and the effect of dyes have likewise yielded no convincing evidence concerning the nature of viruses. Thus it appears that a number of tests with chemical and physical agents have been devised as criteria for the presence of life or to define the nature of viruses, but no one of them has been found satisfactory.

Spontaneous generation of viruses. The origin as well as the nature of viruses constitutes a question of interest. The intimate relation between these active agents and their host cells has induced more than one investigator to view the host cell as the source or origin of viruses. Indeed, reports of experimental work have appeared leading to claims that normal cells have been induced to manufacture certain viruses. According to Carrel (156, 157), minced chick embryo mixed with tar, indol, or arsenic and injected into normal chickens in a small percentage of instances gives rise to tumors resembling Rous' sarcoma no. 1 and transmissible by cell-free filtrates. Fischer (163) by treating cultures of normal cells with arsenic obtained on one occasion a filterable agent capable of causing tumors. Carrel was unable to confirm Fischer's work. Murphy (52, 167), by means of a method the details

of which have not been described, reported that he was able to extract a filterable tumor-producing agent from the gonads of normal-appearing Plymouth Rock roosters. Recently, Hadley and his co-workers (166) stated that it is possible to obtain bacteriophage from normal bacterial cultures by means of enforced dissociation. Although no worker in this field has claimed to have generated living organisms from inanimate matter, it appears that a few believe that they have by certain manipulations induced cells to yield substances which possess some of the attributes of life, notably that of increasing without limit.

The observations described above are suggestive, and, if confirmed and found to warrant the interpretation given them by Carrel, Murphy, Fischer, and Hadley, will prove to be of fundamental biological importance. Unfortunately, however, all of the experiments yielding the observations referred to were conducted in laboratories where workers were actively engaged in the study of agents similar to those supposedly brought into existence. In such laboratories and with such materials it is always difficult for one to rule out the possibility of contaminating normal animals, tissues, bacteria, emulsions, and filtrates. This fact has long been appreciated by workers in vaccine virus[4] laboratories and it delayed the acceptance of the experimental transformation of smallpox virus into vaccine virus. Therefore, experiments of the nature described should never be conducted in rooms used for the study of agents similar to those for which a search is being made. The workers who believe that they have induced viruses to come into existence have not excluded the possibility of the preëxistence of latent viruses or of small amounts of virus in the supposedly normal embryos, gonads, chickens, and bacterial cultures utilized in the experiments. This possibility is emphasized by Flexner's (164) work on poliomyelitis, for he was able to demonstrate the presence of virus in the nasal washings from normal contacts. The possibility outlined is further emphasized by Andrewes and Miller's (155) experience with Virus III in rabbits, by Cole and Kuttner's (158) work with the salivary-gland virus in guinea pigs, and by the work upon virus carriers in general among animals, plants (168) and bacteria.

IMMUNITY. Most virus diseases lead to a marked and lasting immunity in recovered hosts. Not only are the hosts refractory to reinfection, but in their sera antibodies capable of neutralizing the viruses are

[4] See Nicolau, S. and L. Kopciowska. Sur la vaccine spontanée épizootique du lapin, Compt. Rend. Soc. Biol., 1931, cviii, 757.

demonstrable. What bearing have these facts upon the nature of viruses? In the first place, it is certain that viruses are highly antigenic. Furthermore, if our concept of the nature of antigens is correct, the viruses are proteins or are closely linked to proteins. Moreover, the agents are not only antigenic but they give rise to antibodies different from those excited by proteins of the host cells. This is true even of the bacteriophage (188). These facts have been adduced as evidence of the exogenous rather than the endogenous origin of the viruses. Thus the antigenic nature of viruses appears to be prejudicial to the idea that they are products of cellular activity. The notion, however, that a lifeless agent may be injurious to the cell creating it and that it may induce immunological responses independent of those excited by the cell, loses some of its fantastic qualities when one considers the well-known facts that lens protein is not species specific but organ specific and that sympathetic uveitis in the uninjured eye is caused not by microörganisms but by the reaction of the body to substances derived from injured cells of the other uveal tract.

In addition to the neutralizing antibodies, whose mode of action is not clearly understood, complement-fixing antibodies and antibodies causing flocculation in virus emulsions have been described. Schultz and his associates (191–195) contend that the latter types of antibodies are not excited by viruses and that the immunological phenomena in virus maladies are comparable to those induced by toxins. In spite of their contentions, sufficient evidence has been adduced by different workers to make it more than likely that certain virus diseases lead to the production (176, 177, 180, 199) of the antibodies mentioned. Furthermore, Ledingham (184) has recently demonstrated that Borrel bodies in fowl-pox and Paschen bodies in vaccinia are specifically agglutinated by antifowl-pox and antivaccinal sera respectively. The results of these experiments indicate to Ledingham that the elementary bodies are living organisms and represent the virus. There is no reason to doubt that specific agglutinations of the bodies occurred in the manner described by Ledingham, and one cannot deny that such a phenomenon is presumptive evidence of the organismal nature of the bodies. Yet one dare not say categorically that his experiments are unequivocal evidence that the elementary bodies represent virus alone, because it has been shown by Jones (182, 183) that collodion particles treated with a variety of proteins and then thoroughly washed are specifically agglutinated by the proper antisera. Thus, the Borrel and Paschen bodies without being organisms yet having virus adsorbed on them

might nevertheless be specifically agglutinated by appropriate antiviral sera.

Gye (181) states that Rous virus repeatedly injected into alien hosts excites two groups of antibodies, one of which acts on the virus itself, while the other operates on the "specific factor" derived from the host cell. According to him, either set of antibodies inactivates the virus. This fact is offered by him as further evidence of the dual nature of the causative agent of fowl tumors. Murphy (189) and Sittenfield (196–198) have reported the presence in Rous' sarcoma of a substance that inhibits the action of the etiological agent, and the first mentioned worker is of the opinion that the "inhibitor" differs from ordinary virus antibodies. The presence of this "inhibitor" together with other phenomena has induced Murphy (220) to believe that immunity to the Rous agent is unlike that observed in virus maladies and lends evidence to his view that the Rous agent is not a virus. Inhibiting substances, however, have been obtained from tissues infected with viruses, for example, a substance restraining the action of rabic virus has been demonstrated by Marie (186) in the brains of rabid animals. Furthermore, Andrewes' (172, 173) work appears to indicate that the immune responses excited by the filterable agents of fowl tumors may not be unique and may possess much in common with those encountered in other virus diseases.

From what has been said, it is obvious that immunological phenomena are playing an important rôle in discussions concerning the nature of viruses. As yet this method of approach has not brought us to a definite solution of the problem.

CONCEPTS OF THE NATURE OF VIRUSES. A review of the data by means of which one arrives at a concept of the nature of viruses has been presented. Now it will be interesting to see what notions certain workers have concerning some of them.

Beijerinck (202) considers the virus of mosaic disease to be a living contagious fluid; Woods (228), an oxidizing enzyme; Goldstein (212), a protozoal parasite; Vinson (67), an inanimate chemical substance. Most workers, however, believe that it is a minute living organism.

Högyes (216)[5] is of the opinion that the incitant of rabies is an enzyme or, "alternatively, that the tissues themselves might spontaneously become virulent as the result of changes in their chemical composition." At one time Remlinger[6] said, "The rabies virus, which is at once filter-

[5] See A. C. Marie (218), p. 12.
[6] See A. C. Marie (218), p. 22.

able, diffusible and capable of reproducing the disease from case to case, appears to occupy a place midway between the microbes and the diastases." Recently, however, he (221) has published an article on the evolution of the *parasite* of rabies. Levaditi (118) and others (121, 225) have presented evidence in favor of the idea that the causal agent is a protozoon. The majority of investigators hold the concept that the incitant is a living organism whose nature is not definitely known.

Numerous workers believe that the bacteriophage is an inanimate agent, while others are convinced that it is a living organism. Ideas, however, concerning the nature of the inanimate transmissible substance or the animate organism vary. For details of the different concepts one is referred to papers by Twort (226, 227), d'Herelle (103), Bordet (203), Bronfenbrenner (95), Burnet (206), and Hadley (165, 166).

The incitant of fowl-pox has been described by certain investigators as a protozoal parasite. Sanfelice (222, 223) suggested that it is a nucleoproteid poison manufactured by infected cells. Borrel (109), Goodpasture (124, 125), and Ledingham (184) hold that it is a minute coccoid organism capable of regeneration in parasitized cells.

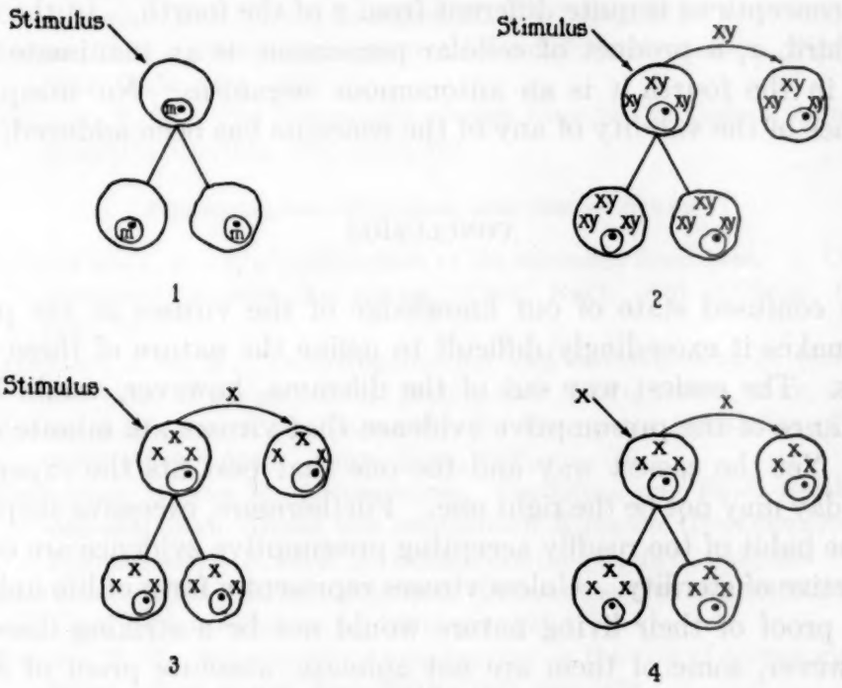
Rous and others are prepared to entertain the idea that the causal agent of Chicken Tumor No. 1 is animate. Gye (215) believes that it consists of two factors, one of which is a living exogenous organism, the other an inanimate specific factor derived from infected cells. Murphy (52, 55), at one time, spoke of the Rous agent as an enzyme-like substance. Recently, however, he (220) has compared it to filterable substances capable of transforming melitensis (204, 205) into paramelitensis organisms and of converting one type specific pneumococcus (201) into another type specific form. In regard to the matter he says (220), "Thus we have a group of agents, products of specialized cells capable of conferring the peculiar type quality to undifferentiated cells of the same species which, in turn, may produce the active factor and transmit this to their descendants." For this type of agent he proposes the name transmissible mutagens.

Sufficient ideas concerning the nature of viruses have been cited to illustrate how radically some differ from others. Many of them, particularly the ones dealing with the origin and reproduction of inanimate substances that behave in a manner similar to that of living organisms, lack precision. In a general way, however, the different concepts can be arranged in groups and it seems advisable to state and to portray diagrammatically several of the popular ones.

According to one conception, certain stimuli produce changes within

cells that are inherited by daughter cells. Once the mutations occur, cells of the new type continue to be formed though the stimuli disappear. No agents separable from the cells are demonstrable, and immunological phenomena in this type of disease differ from those observed in virus maladies. Ordinarily this idea of the causation of disease and the concepts concerning the nature of filterable viruses are not grouped together. Yet in some respects they are not dissimilar and many hold the view that malignant neoplasms arise in some such way. See figure 1.

Another notion is that appropriate stimuli induce normal cells to



Figs. 1, 2, 3 and 4

make a substance x which is closely bound to parts y of the cells. Thus an xy complex is formed. This complex, separable from the cells, yet capable of inciting its own production by them, either passes directly into daughter cells, or, having become extracellular, enters another set of normal cells. The xy complex is antigenic, and cells freed from it presumably become normal again. See figure 2.

Still another idea is that certain stimuli incite normal cells to produce a substance x which is not closely bound to parts of the cells. X, separable from cells, yet capable of impelling its formation by them, either passes directly into daughter cells, or, having become extra-

cellular, enters a new group of normal cells. X is antigenic and cells freed from it presumably become normal again. See figure 3.

Finally there is the concept most generally held that x is not a product of the perverted activity of cells but is a minute living organism. X enters cells, multiplies, produces disease, is separable from cells, and is antigenic. Cells freed from it presumably become normal again. At times, x is absorbed by particles y of host cells and evidences of an xy complex are obtained. See figure 4.

For practical purposes it makes little difference which one of the last three concepts is accepted. Theoretically, however, x of the second and third conceptions is quite different from x of the fourth. In the second and third, x, a product of cellular perversion, is an inanimate agent, while in the fourth it is an autonomous organism. No unequivocal evidence of the validity of any of the concepts has been adduced.

CONCLUSION

The confused state of our knowledge of the viruses at the present time makes it exceedingly difficult to define the nature of these active agents. The easiest way out of the dilemma, however, would be the acceptance of the presumptive evidence that viruses are minute organisms. Yet the easiest way and the one that best fits the experiences of the day may not be the right one. Furthermore, excessive skepticism and the habit of too readily accepting presumptive evidence are equally productive of sterility. Unless viruses represent a form of life unknown to us, proof of their living nature would not be a striking discovery. If, however, some of them are not animate, absolute proof of such a fact would be of fundamental biological importance. Therefore, care should be exercised that immoderate skepticism on the one hand, and the mental satisfaction secured by accepting presumptive evidence on the other, do not dull our efforts to obtain a better understanding of the viruses, some of which may be minute organisms, while others may represent forms of life unfamiliar to us, while still others may be inanimate transmissible incitants of disease. In any event, we are face to face with the "infinitely small in biology," and, if there be a sharp demarcation between life and death, then scientists, investigating the nature of viruses, are working near the line that separates infinitely small living organisms from inanimate active agents.

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THE EFFECT OF MUSCULAR FATIGUE UPON RESISTANCE

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The prevailing view is that muscular fatigue lowers resistance and is a predisposing factor to infectious diseases, especially with regard to respiratory infections. This view has partly arisen from the fact that a sensation of fatigue, due to the early stages of an infection, frequently precedes the recognition of the disease. But quite apart from this there is a generally accepted idea that fatigue favors the onset of infectious diseases or makes possible the activation of an already present but hitherto latent disease. Comparatively little experimental work has been done to test this view scientifically. That such a problem is difficult or almost impossible to solve at present is obvious from the fact that our knowledge of what constitutes resistance is still very limited. Furthermore, the problem is complicated by the fact that the conception of muscular fatigue includes such varied conditions as the fatigue resulting from a single maximal effort, as in competitive athletics, and fatigue which has accumulated over long periods of time from heavy industrial work.

The available evidence dealing with the effect of muscular fatigue on resistance falls into three groups: 1, experiments testing the effect of one or more periods of exhaustive exercise on the general resistance of animals to experimentally induced infections, as indicated by the severity and outcome of the infection; 2, sickness and mortality records of athletes and men engaged in heavy industrial work; 3, experiments concerning the effect of exhaustive muscular exercise on the various basic defense mechanisms of the body tissues and fluids.

1. *The effect of muscular fatigue on the resistance of animals to experimentally induced infections.* Various experimenters have sought to study the effect of muscular fatigue on the course of experimentally induced infections in the usual laboratory animals. With the exception of De Sandro, who fatigued his animals by faradization, all of the experiments have been carried out in a similar manner. This consisted of subjecting animals to exhaustive exercise in rotating drums on one or more

days either immediately before or following inoculation with a culture of an organism. The resistance of the animal was considered to have been lowered by fatigue when the resulting infection caused death more rapidly and to a greater extent among the fatigued animals than among another group of animals similarly inoculated but not exercised.

Charrin and Roger (1890) found that fatigue, following subcutaneous inoculation with the bacilli of anthrax or symptomatic anthrax, lowered the resistance of rats to infection with these organisms. These authors also claimed that fatigue lowered the resistance of animals to the invasion of organisms through skin eruptions and possibly through the intestinal wall on the experimental evidence that normal guinea pigs, not inoculated, died upon being exercised, showing organisms in their blood and tissues.

De Sandro (1910) reported that a small group of fatigued dogs, rabbits and guinea pigs died sooner than their controls when inoculated subcutaneously with typhoid toxin.

The experiments of Abbot and Gildersleeve (1910) showed a lowered resistance to intravenous inoculation with streptococci pyogenes in a few rabbits fatigued before inoculation. All of the fatigued rabbits died prior to the controls, some of which survived. When, however, staphylococcus pyogenes aureus was the organism used, injected intravenously or subcutaneously, rabbits similarly fatigued before inoculation reacted exactly like their controls. If these organisms were introduced prior to exercise, however, fatigue was found to lower the resistance of rabbits so that all of the fatigued animals succumbed, whereas the controls survived.

A series of experiments by Spaeth and his students, however, did not confirm these earlier results. Oppenheimer and Spaeth (1922) showed that fatigue did not lower the resistance of rats to tetanus toxin. Furthermore, they found that one to three periods of fatigue, whether preceding or following intraperitoneal injection of a culture of type 1 pneumococcus, but more especially when preceding infection, definitely increased the resistance of rats to that infection. Of 61 fatigued animals, 29 per cent of those fatigued before and 57 per cent of those fatigued after inoculation died as compared with 76 per cent among the 17 controls. Exactly similar results were obtained in guinea pigs by Nichols and Spaeth (1922).

Knauf (1928) repeated the experiments of Oppenheimer and Spaeth on rats fatigued after inoculation, using the same organism, cultured and injected in the same manner, with results, however, directly contradic-

tory to those obtained by Spaeth. A decreased resistance in the fatigued animals was evidenced by a higher mortality (70 per cent of the 30 fatigued rats died as compared with 40 per cent of an equal number of controls), as well as by a shorter mean time of death.

Bailey (1925) studied the effect of muscular exercise on the resistance of a large number of rabbits to intratracheal injection with type 1 pneumococcus. When the rabbits were fatigued one to three periods before or after inoculation, their resistance to the organism was markedly lowered (70 per cent of the 60 fatigued rabbits died with an average survival time of 2.2 days as compared with 46 per cent of the controls with an average of 3.5 days). This increased susceptibility was much greater when infection preceded the exercise. On the other hand if the rabbits were fatigued from six to twelve periods on alternating days before inoculation, during which time they became trained to such exercise, they were found to be more resistant to intratracheal infection with pneumococci than were the controls.

The above experiments, with the exception of those of Charrin and Roger, are subject to the criticism that the organisms were introduced into the animal in a manner quite foreign to the natural path of entry. It has, therefore, been claimed that such experiments are a poor test of the effect of fatigue on the normal bodily defenses. With this in mind Bailey (1929) repeated his previous experiments, this time introducing the organisms into the nasal passages, as the normal mode of entry of the pneumococcus is probably through the respiratory mucous membrane. The results obtained entirely confirmed and extended his previous conclusion. That rabbits subjected once or twice to exhaustive exercise preceding or following intranasal inoculation were less able to resist infection was shown by a more frequent invasion of the blood stream by the organism, more rapid and severe infection, a shorter mean time of death and a greater mortality in the fatigued rabbits than among the controls (90% of the 21 fatigued rabbits died as compared with only 25% of the controls). This harmful effect of fatigue was again much more evident when inoculation preceded the exercise. Fatigue, however, either before or after inoculation, did not break down the high resistance of immunized rabbits to infection with type 1 pneumococcus.

Boycott and Price-Jones (1926) employed Gaertner's bacillus as the infecting agent, this being a natural parasite of the rat. When the organism was introduced subcutaneously or intraperitoneally, that is, by an unnatural path of entry, fatigue was without effect on the resulting mortality. If, however, a culture of this bacillus was fed to the rats,

the resistance of the animals was found to be markedly reduced by fatigue, as evidenced by extensive illness and deaths among the fatigued animals as compared with practically no reaction among the controls. In another group of experiments fatiguing rats at intervals over a period of eight weeks was found to be ineffective in breaking down their natural resistance to human and bovine tuberculosis.

Merrill and Howe (1928) used, as their infecting agent, B. pyocyaneus introduced intraperitoneally assuming that, as this organism normally enters through the skin, such a method of inoculation simulated the normal mode of entry. Fatigue, especially when following inoculation, was again found to lower the resistance of rats, as seen by illness and death among the fatigued rats whereas the controls showed no effects of the inoculation. In another group of rats regular systematic exercise every other day for six weeks previous to inoculation apparently increased the resistance of the animals, so that fatigue following inoculation exerted a less injurious effect. This appears to confirm Bailey's results showing an increased resistance with repeated exercise.

In summing up the results of these experiments, undertaken to test directly the effect of fatigue on resistance in animals, one cannot feel convinced that they have furnished very much conclusive evidence. In the first place, many of the experiments have been carried out on very small numbers of animals. Anyone who has had any experience with inoculation experiments is aware of the variation in resistance which exists even among comparable groups of normal animals. Hence a few deaths more or less in a small group, although appearing, when calculated as percentage, to give a significance to the results, may be well within the limits of the normal variations. And, in fact, the normal level of resistance of small laboratory animals to the various organisms, toxins, etc., inoculated by the various methods, has not been sufficiently well established to serve as an adequate basis of comparison for the fatigue experiments. In the second place, the data offered by the different experimenters are somewhat contradictory.

However, taking all the results into consideration, the evidence would seem to indicate that one or two periods of exhaustive muscular exercise following immediately after inoculation of the animal with foreign organisms leads to a more frequent appearance of an infectious disease and induces a greater and more rapid fatality. Fatiguing muscular exercise over one to three periods preceding inoculation also appears to lower the resistance of the animal to infection but not to the same extent as when the exercise follows the infection. There is some suggestive

evidence that repeated exhaustion may enhance resistance although this point is in need of more experimental work.

2. *The effect of exhaustive muscular work on resistance of man to infectious diseases.* It is almost impossible to ascertain the effect of any one factor, such as fatigue, on the resistance of man to infectious diseases. The only available methods of obtaining information on this problem are first, to compare the sickness records of men engaged in strenuous athletics with similar records of men not engaged in athletics; and secondly, to compare the morbidity and mortality statistics obtained from industries where the men are engaged in heavy muscular work with similar statistics of men exposed to the same conditions but engaged in light muscular work. A few attempts to collect some such evidence on this subject have been made. The results will be briefly reviewed here, more with the idea of pointing out the difficulties of such a problem and how little information is available rather than in the hope of presenting any conclusive evidence on the effect of fatigue on resistance.

With regard to the effects of athletic exercise, Cowles (1918) has presented evidence in support of the results reported in animals. He claimed that acute muscular fatigue, due to violent exercise like rowing or running in competitive sports, following mild respiratory infections is a contributing cause of pneumonia. His view was based first on the fact that four to five per cent of the pupils in a boys' school developed pneumonia each year over a period of three years, which was confined to the boys given to athletic tasks and sports and was not found at all among the faculty or employees of the school nor among the pupils of a nearby school who limited themselves to everyday activities, and secondly, on the fact that no cases of pneumonia occurred among the pupils over a period of five years when the boys subject to, or convalescent from, mild respiratory infections were shielded from fatigue.

Dublin (1928), who studied the longevity of almost 5000 college athletes who graduated in 1905 or earlier, showed that this group had a lower death rate than selected insured men. This does not necessarily indicate, however, that college athletics lead to an increased resistance to infections or a longer life because the death rate includes all causes, degenerative as well as infectious diseases, and college men are a more select group than insured men. Analysis of the causes of death among this group showed that tuberculosis was responsible for 16 per cent of the reported deaths under 45 years of age and that pneumonia and typhoid also ranked high. It is interesting to note that the Actuarial Society records (1900–1915) show that tuberculosis accounted for approximately

32 per cent of the total deaths between the ages of 20–29 years and 21.7 per cent for the 30–39 year age group among men in their sixth and subsequent insured years. Typhoid and pneumonia were the next most frequent causes of death in their tables. Although these figures are not exactly comparable to Dublin's, nevertheless they indicate that tuberculosis was not a more frequent cause of death among athletes than among normal insured men of the same age group.

Vernon (1921a) studied the effect of fatigue on the sickness records of munition workers and reported a close parallelism between the percentage time lost due to sickness and the number of weekly hours of labor, as shown by the following figures:

Men	Hours of work	63¼	62	54
	Percentage time lost from sickness	7.0	5.7	4.0
Women	Hours of work	62	54	40
	Percentage time lost from sickness	6.4	4.3	3.1

How much of the sickness was due to infectious diseases and how much to other conditions is not recorded. Also it must be remembered that longer hours of labor mean a longer period of exposure to other industrial conditions predisposing to disease in addition to fatigue.

Vernon (1921b) also studied the effect of fatigue on the health and longevity of men engaged in the iron and steel industry for the years 1913–1918, an industry demanding the most exhaustive type of muscular work under the most unfavorable temperature conditions for 11 to 12 hours per day. He concluded that, with the possible exception of the steel melters, the fatigue of this heavy work as a rule showed but little direct effect on the sickness and longevity of men of good physique. However, the steel melters, those men engaged in the most exhaustive of all work, lost 23 per cent more days of sickness than the average for all steel workers, and showed an increased mortality of 26 per cent above the average mortality of the whole group of steel workers, and 20 per cent greater than that of all males. Vernon believed that, as most of this excess sickness and mortality was attributable to respiratory diseases (exclusive of tuberculosis), it was largely due to the unfavorable temperature conditions but he considered that fatigue was probably a factor since other diseases also showed some excess.

Collis (1926) claimed that fatigue due to overstrain in industry is a factor in the high tuberculosis mortality rate occurring in late middle life because (1), the rate was higher in industrial than rural counties; (2), the rate for males was higher than that for females, who led a less strenu-

ous life; and (3), the mortality curve in the late middle age period for all males coincided with the curve for those males known to be exposed to factors predisposing to tuberculosis, thus indicating that the general curve showed a factor predisposing to tuberculosis, probably fatigue. Because of the many factors in industrial employment other than fatigue, which predispose to tuberculosis, the above argument can hardly be considered as offering much evidence concerning fatigue and resistance.

It cannot, therefore, be said that there is much, if any, evidence to show that muscular fatigue lowers the resistance of man to infectious diseases. The evidence presented by Cowles appears to support the results obtained on animals, showing a lowered resistance when fatigue follows inoculation, but such single observations are, of course, not significant. It is to be hoped that further statistical studies on athletes will lead to some more definite evidence on this subject, such studies, for example, as comparing the sickness and mortality records of college athletes with those of their unathletic classmates. But even here the value of such evidence would be limited for, although exhaustive exercise may be without effect on the resistance of the physically well trained man, it may have a marked effect on the resistance of a man unaccustomed to physical exertion. It is doubtful whether industrial statistics will ever yield any information; there are so many other factors in industrial conditions affecting resistance that it is impossible to judge the effect of a single factor such as fatigue. Furthermore with the invention of modern machinery and shorter hours, industrial processes causing muscular exhaustion are rapidly diminishing.

3. *The effect of muscular fatigue on the defense mechanisms of the body.* If muscular fatigue leads to a lowered resistance to infectious diseases, then this would seem to imply that those mechanisms of the body which protect it from foreign organisms have been altered by the exhaustive muscular work. The body defends itself against micro-organisms primarily by preventing the invasion of them through the skin and mucous membranes. If, however, this occurs the tissues react to destroy the invading organisms by the production of antibodies and by the process of phagocytosis. As the defense mechanisms of the body are still not very well understood, attempts to study the effect of exercise on them have yielded little information.

Ficker (1906) has presented the only evidence concerning the effect of muscular fatigue on the permeability of a mucous membrane to bacteria. He claimed that exhaustive exercise increased the permeability of the

intestinal wall to "Roten Kieler," which was fed to dogs, and, also, to the normal intestinal bacteria such as B. coli and B. proteus, because such bacteria were found in cultures of the blood and tissues taken from fatigued dogs whereas similar cultures from tissues of control dogs were negative. The amount of invasion of the organs, however, showed no parallelism to the number of hours of exercise nor the apparent degree of fatigue. Charrin and Roger, quoted above, believed that their experiments also indicated a greater permeability of the intestinal wall in fatigued guinea pigs. Boycott and Price-Jones, on the other hand, concluded that an increased permeability of the intestinal wall to Gaertner bacilli, administered orally, was not evident in their fatigued rats because these bacilli were recovered in splenic cultures from the controls as well as from fatigued animals.

Nothing is known concerning the effect of muscular fatigue on the defensive power of the respiratory mucous membrane. In fact, the effect of exhaustive exercise on the vascular condition of this tissue and its secretion has not been studied and, furthermore, the conditions of the mucous membrane which favor development and invasion of microorganisms are also not well known.

If fatigue lowers resistance, one would expect a decrease in the normal antibody content of the blood or in the capacity of the tissues to produce antibodies. Experiments on this phase of the problem have, however, yielded conflicting results.

With regard to the effect of fatigue on antitoxins, Zlatogoroff and Kostereff (1931) found that, following a march of 40 km., the negative reactions of Shick and Dick, which connote the presence of antitoxin, became positive in a large group of men as shown by a comparison of the tests made five days prior to and again immediately following the march. Ten days of rest following this resulted in a condition again approaching that obtained prior to the exhaustive exercise.

With regard to the effect of fatigue on the formation of agglutinins, a decreased ability to produce typhoid agglutinins, following exhaustive exercise, was reported by Lara and De Jesus (1930), by Scalfati (1912), and De Sandro (1910). Trommsdorff (1906) obtained similar results when guinea pigs were repeatedly fatigued but opposite results when they were exercised only once. Bailey (1929) reported that fatigue increased not only the quantity but also the rate of agglutinin formation in rabbits when exercised before and several times after inoculation. No consistent variation in the agglutinin production as a result of fatigue was found by Vallardi (1912) nor in the normal content by Ficker.

Except for the experiments of Lara and De Jesus, the observations were made on small groups of animals.

With regard to the effect of fatigue on the bactericidal power of the blood, Trommsdorff observed a lowered bactericidal power in guinea pigs exercised six to eleven days after inoculation with B. typhosus but an increased power when the animals were exercised only once. On the contrary, Ceni (1893) reported a diminution (in response to the same organism and B. anthrax) in the bactericidal power of the blood in sheep and dogs which were subjected to work of short duration (one to one and one-half hours), but an augmentation, especially in dogs, if fatigue was prolonged three or more hours. No decrease in the production of typhoid bacteriolysins was found by Vallardi in guinea pigs, and no decrease in the normal bactericidal power by Ficker in dogs, as a result of exercise, but a diminished production of bacteriolysins was reported by De Sandro in animals fatigued by faradization.

Trommsdorff found that the formation of hemolysis in the blood of immunized guinea pigs was retarded by intense fatigue while Ficker observed no decrease in the normal hemolytic power of dogs following fatigue.

Various experimenters have endeavored to show an effect of fatigue on the alexine content of the blood. No decrease in the alexine content was found in the serum of fatigued animals by Bailey (1929), Vallardi, and Trommsdorff. The latter, however, claimed that when the alexine in the blood serum of guinea pigs was destroyed, its normal rapid regeneration was prevented by subjecting the animals to fatiguing exercise. Dobrowolny (1930) reported a decrease in alexine content following complete exhaustion in trained rabbits but no decrease or even an increase in untrained rabbits or if the exercise was not exhaustive. With regard to the alexine content in the blood of man following exhaustive athletic exercise, Deutsch and Hermann (1930) reported no consistent variation in either direction. Huntemüller (1929), on the other hand, reported a marked fall in the alexine content in the blood of the Olympic contestants following competitions in the winter sports. The results among the summer contestants were extremely irregular. He believed that a condition of overtraining previous to the competition led to a diminished complement content of the blood. There is, therefore, no conclusive evidence to show that muscular fatigue causes a decrease in the normal complement content of the blood. Moreover, even if a decrease could be demonstrated it could not be claimed that this was evidence of a lowered resistance. Hyde (1923) has shown that the

amount of complement present in the serum is no indication of resistance since under normal conditions complement-deficient guinea pigs were found to be equally as resistant to infection as normal guinea pigs.

With regard to the effect of fatigue on the opsonins of the blood and the phagocytic power of the leukocytes, a closer agreement is found among the reported results. Abbott and Gildersleeve obtained a progressive reduction in the opsonic index, in relation to streptococcus pyogenes, amounting to 25–60 per cent, in a number of rabbits with repeated periods of severe muscular exercise. Ellett (1906), using the tubercle bacillus as the test organism, reported a fall in the opsonic index of two men following strenuous athletic exercise. A diminution in the phagocytic power of the blood against typhoid bacilli was reported by Scalfati and Di Macco (1920) in rabbits and guinea pigs following fatiguing exercise. Prawditsch-Neminsky and Shelinskaja (1930) obtained a decrease of the phagocytic power of blood, in reference to the tubercle bacillus, in children following physical work, attributed to a decrease in lipase, in turn dependent on a decrease in the number of lymphocytes, but an increased phagocytic power in relation to granulated rice starch. Trommsdorff observed a diminished invasion of leukocytes into the peritoneum, a retarded process of extra-cellular dissolution and a lowered phagocytosis by the leukocytes in the peritoneal fluid in a few fatigued guinea pigs following intraperitoneal injection of foreign erythrocytes. Since it is known that in many infections the resistance is roughly proportional to the opsonic content of blood and since the phagocytic power of the leukocytes depends largely on the presence of opsonins in the blood, the above evidence seems to indicate a lowered resistance as a result of fatigue.

Ronzani (1907) studied the effect of fatigue on the bactericidal properties of the lungs believing this to depend on phagocytosis, the bactericidal power of the blood, the secretion of lung epithelium, lymph flow, etc. He claimed that this protective ability of the lung was decreased by fatigue as evidenced by the fact that guinea pigs fatigued following the inhalation of B. prodigiosus showed the presence of bacteria in lung cultures even after 72 hours, whereas unexercised guinea pigs were normally able to eliminate all the bacteria by the end of 48 hours. Bailey's (1925) results appeared to confirm this. He reported that 40 per cent of the fatigued rabbits showed marked pulmonary lesions following intratracheal inoculation with type 1 pneumococcus as compared with only 20 per cent of the controls. Since this was true even when fatigue preceded inoculation it would appear to be due to a lowered

bactericidal power of the lungs rather than to a greater dissemination of the bacilli due to the more rapid breathing during exercise.

According to many investigators the defense of the body against foreign organisms rests more upon the activity of the cells of the reticulo-endothelial system and the white blood cells than upon the humoral antibodies. In fact, many observations favor the theory that antibodies are the product of the reticulo-endothelial cells, although final proof for this is lacking (Jaffé, 1931). To the writer's knowledge, practically nothing is known concerning the effect of muscular fatigue on the reticulo-endothelial system. The effect of exercise on the number of white blood cells in the circulation, however, is comparatively easy to study and so has been the subject of many investigations especially on man, but the significance of the results in terms of resistance is not at all clear. Muscular exercise produces a marked increase in the total leukocyte count, so called myogenic leukocytosis, the increase varying from 20 per cent to well over 100 per cent, depending on the intensity, duration, and condition of the exercise and on the subject. Egoroff (1926) reported an increased white cell count of 2000 to 5000 with runs of 5 to 10 km. and 14,000 following a Marathon race of 40 km. Much greater increases have been reported by Garrey (1929), for example, an increase of 14,000 following a 100 yard dash. Differential counts have also been made. During the early period of exercise or immediately after exercise of short duration there is an absolute and relative rise in lymphocytes which, according to some observers, is followed within one to two hours after cessation of exercise by an increase in neutrophiles and a relative fall in lymphocytes. If the strenuous exercise is of long duration this initial rise in lymphocytes is followed by a marked, absolute and relative rise in neutrophiles, and a marked fall in the percentage of lymphocytes during the later periods of the exercise. (Egoroff, 1926; Herxheimer, 1928; Schneider and Havens, 1915; Hartman and Jokl, 1930, and others.) Egoroff reports that an absolute lymphopenia and a relative and occasionally absolute lack of eosinophiles results from work of long duration and that the altered blood picture may still be present for hours after the end of exercise. Edwards and Wood (1932) also observed an absolute lymphopenia appearing within an hour after exercise and returning to normal within 3 to 6 hours.

Merely to know the changes which occur in the number of circulating cells, however, is no indication of their significance in regard to resistance. The essential question is whether an increased number of circulating neutrophiles and a relative and possibly absolute decrease of circulating

lymphocytes, which occur with exhaustive exercise of long duration, indicate a lowered or enhanced resistance. There is some evidence that a low lymphocyte count indicates a lowered resistance under certain conditions. Murphy (1926) has shown that, if the number of lymphocytes in the circulating blood is experimentally reduced, the resistance of mice to tuberculosis and cancer is lowered. Doan and Sabin (1930) observed that a low ratio of lymphocytes to monocytes in the peripheral blood of tuberculous animals is correlated with low resistance, and that a naturally low lymphocyte-monocyte index in normal healthy rabbits previous to infection with tuberculosis can be correlated with the subsequent development of an acute fulminating tuberculosis. No observations have been reported concerning the effect of exercise on the monocytes. Furthermore, whether the low lymphocyte count with exercise is due to the same fundamental changes as those causing the low lymphocyte counts of Murphy and Sabin is questionable. It is questionable, also, whether the fairly temporary decrease in the relative number of lymphocytes, such as occurs after exhaustive exercise of long duration, could affect significantly the course of such a slowly developing type of infection as tuberculosis.

What the marked increase in neutrophiles with exercise signifies with regard to resistance is not known. Infection with organisms such as pneumococcus or streptococcus is practically always accompanied by an increase in circulating neutrophiles which is taken to be a sign of active resistance. This does not imply, however, that a high circulating neutrophile count, due to causes other than the infection, indicates an increased resistance. Bull and McKee (unpublished) found in a group of fifty-seven normal rabbits that those showing a relatively high total white count before infection were more susceptible to pneumococcus infection than those with lower counts. However, these experimenters feel that, since the normal leukocyte count varies enormously, data from such a small group of rabbits are of little significance. In addition, the proportion of neutrophiles is not known as differential counts were not made.

With regard to the effect of fatigue on the white blood cells, it is not only important to know the effect of exercise on the number of circulating blood cells and its significance, but also the effect of exercise on the ability of the leukocytes to phagocytize foreign organisms. Since this depends largely on the presence of opsonins in the serum the little evidence on this point was presented above in discussing the effect of fatigue on opsonins.

The evidence, therefore, in regard to the effect of muscular fatigue on the antibody content of the blood and the production of antibodies is very contradictory; the majority of the experiments indicate a decrease in antibodies but many negative and even reverse results have been obtained. Much more experimental work on larger numbers of animals will have to be done before any conclusions can be drawn. Nothing is known regarding the effect of fatigue on the reticulo-endothelial system, and the significance of the observed changes in the number of circulating blood cells is not at all clear.

Discussion. The experiments reviewed above appear to indicate that exhaustive exercise just preceding or immediately following infection, but more especially the latter, predisposes the animal to a more rapid and fatal attack of the infectious disease. From our present knowledge it is impossible to say that this is confirmed in man although there is a very general belief that it is true. If fatigue does lower resistance, it should be expected that some demonstrable effect of exhaustive muscular exercise would be evident on those mechanisms, serological and cellular which are known to protect the body against infections, but little experimental evidence has been reported on this phase of the problem and this is largely contradictory. But even if it were proven that fatigue lowers resistance, that antibody production is reduced or that the phagocytic ability of the cells is diminished as a result of muscular fatigue, there would still remain the problem of how exhaustive muscular exercise brings about these changes.

There are various ways in which exhaustive muscular exercise may affect the resistance of the body. The increased circulation of the blood and especially of the lymph, which occurs in exercise, must lead to a more rapid and extensive dissemination of the organisms from the point of entry, so that in place of a more or less localized infection a more generalized infection would result, thus throwing a greater burden upon the defense mechanisms. Bailey, in fact, stated that local infections were more apparent, while invasion of the blood stream occurred less rapidly and frequently in control animals than in the fatigued. If the organisms enter through the respiratory tract, the increased rate and depth of respiration would lead to a greater dissemination in the lungs. According to Krause (1923) the increased circulation and respiration occurring in intense or prolonged physical effort is one factor in the activation of old, quiescent foci of tuberculosis. So also the amount of absorption of focal toxic substances, and thus the resulting degree of intoxication, depends upon the circulatory and respiratory

activities of the body. It would not seem, however, that the circulatory and respiratory changes can be the entire explanation of the lowered resistance in fatigued animals reported above, because a lowered resistance was also apparent in animals inoculated after cessation of fatiguing exercise, when the circulation and respiration would be rapidly returning to if not already at normal.

Since it was early shown that injection of lactic acid into a muscle favored the development of anthrax, it was assumed that the local production of lactic acid during muscular contraction would also have the same effect (Arloing and Cornevin, 1886; Herzens, 1889). This idea was proved untenable by experiments in which the muscle inoculated with anthrax was stimulated directly with no greater development of anthrax (Roger, 1889), and it has since been shown that the effect of injecting lactic acid was due merely to tissue injury. It is doubtful whether the acid produced during exercise could cause a lowered resistance, because even in severe exercise the pH of the blood is probably not sufficiently altered to affect the activity of the cells concerned in resistance.

There is good evidence that the metabolic condition of the animal, evidenced by a loss in weight, which results from muscular exercise is one factor in the lowered resistance to infection. Spaeth (1925) observed that those guinea pigs which showed a gain in weight following fatigue and infection invariably recovered. He, therefore, concluded that resistance is a function of the anabolic rate following infection and substantiated this theory by showing that young starved guinea pigs and exhausted guinea pigs, when fed immediately after inoculation with pneumococcus, resisted the infection better than normally fed control pigs. According to Ledingham (1931) there is ample proof that the resistance of an animal to both spontaneous and induced infection is profoundly affected by nutritional defects including generally restricted diets as well as those lacking particular vitamines; and that furthermore, the normal irritability of the reticulo-endothelial tissue is very greatly reduced by defects of diet which lead to loss of weight. It would seem, therefore, that the impairment of the metabolism in exhaustive exercise may be one factor which contributes to a lowered resistance to infection but how this occurs is at present not understood.

It is possible also that muscular fatigue may alter resistance by affecting the glands of internal secretion, especially the adrenal cortex, since it has been shown that this gland is closely connected with resistance against infections (Scott, 1924; Jaffe, 1926; Belding and Wyman, 1926; Hartman and Scott, 1931, etc.). That exercise may affect the adrenal

cortex is indicated by unpublished experiments of Hartman (1931) who found an increase in the weight of this tissue in rats after repeated periods of exercise. Furthermore, an abnormal histological appearance of this organ following severe muscular fatigue has been noted by Vincent (1925) and Crile (1921).

Alterations in body temperature with exercise might be considered as a possible factor in the effect of exercise on resistance. This does not seem likely, however, since the experiments of Boycott and Price-Jones "showed that under conditions in which fatigue increases the mortality from infection, the result is the same whether the animals' (rats) temperatures fell or are maintained at the normal level by a high air temperature." Furthermore, Bailey (1929) reported a similar decreased resistance as a result of fatigue in rabbits which showed a rise in body temperature with exercise.

In view of the varying and indecisive character of the results so far obtained one must conclude that muscular fatigue does not exert a dominating influence upon the activities of the defense mechanisms of the body. Its influence must be of a minor character which can be revealed satisfactorily only by controlling the variable conditions that enter into such experiments. The more obvious of these variables are the mode of infection, the individual differences in resistance, and the variations in virulence of the infective organisms. As regards the first it would seem to be essential to establish the infection through the usual portals of entry and by methods similar, as far as possible, to those occurring in nature, so that the normal defense mechanisms may come into action. So far as the other two factors are concerned reliance must be placed upon the use of large numbers of experimental animals and the careful control of the dosage and virulence of the organism employed.

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